DEVELOPMENT OF NOVEL HIGH-RESOLUTION MELTING (HRM) ASSAYS FOR GENDER IDENTIFICATION OF CARIBBEAN FLAMINGO (*Phoenicopterus ruber ruber*) AND OTHER BIRDS

A Thesis

by

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Submitted to the Office of Graduate Studies of Texas A&M University
and the Graduate Faculty of The Texas A&M University – Corpus Christi
in partial fulfillment of the requirements for the joint degree of

MASTER OF SCIENCE

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December 2012

Major Subject: Marine Biology

ABSTRACT

Unambiguous gender identification (ID) is needed to assess parameters in studies of population dynamics, behavior, and evolutionary biology of Caribbean Flamingo (*Phoenicopterus ruber ruber*) and other birds. Due to its importance for management and conservation, molecular (DNA-based) avian gender ID assays targeting intron-size differences of the Chromosome Helicase ATPase DNA Binding (CHD) gene of males (CHD-Z) and females (CHD-W) have been developed. Male (ZZ) and female (WZ) genotypes are usually scored as size polymorphisms through agarose or acrylamide gels. For certain species, W-specific restriction sites or multiplex polymerase chain-reaction (PCR) involving CHD-W specific primers are needed.

These approaches involve a minimum of three steps following DNA isolation: PCR, gel electrophoresis, and photo-documentation, which limit high throughput scoring and automation potential. In here, a short amplicon (SA) High-resolution Melting Analysis (HRMA) assay for avian gender ID is developed. SA-HRMA of an 81-Base Pair (bp) segment differentiates heteroduplex female (WZ) from homoduplex male (ZZ) genotypes by targeting Single-nucleotide Polymorphisms (SNPs) instead of intron-size differences between CHD-Z and CHD-W genes.

To demonstrate the utility of the approach, the gender of Caribbean Flamingo (*P. ruber ruber*) (17 captive from the Dallas Zoo and 359 wild from Ría Lagartos, Yucatán, Mexico) was determined. The assay was also tested on specimens of Lesser Flamingo (*P. minor*), Chilean Flamingo (*P. chilensis*), Saddle-billed Stork (*Ephippiorhynchus*

senegalensis), Scarlet Ibis (*Eudocimus ruber*), White-bellied Stork (*Ciconia abdimii*), Roseate Spoonbill (*Platalea ajaja*), Marabou Stork (*Leptoptilos crumeniferus*), Greater Roadrunner (*Geococcyx californianus*), and Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*). Although the orthologous 81 bp segments of Z and W are highly conserved, sequence alignments with 50 avian species across 15 families revealed mismatches affecting one or more nucleotides within the SA-HRMA forward or reverse primers. Most mismatches were located along the CHD-Z gene that may generate heteroduplex curves and thus gender ID errors. For such cases, taxon and species-specific primer sets were designed. The SA-HRMA gender ID assay can be used in studies of avian ecology and behavior, to assess sex-associated demographics and migratory patterns, and as a proxy to determine the health of the flock and the degree by which conservation and captive breeding programs are functioning.

ACKNOWLEDGEMENTS

Most importantly, I would like to thank my mom and dad for their unconditional love, enthusiasm, advice, and yes, money and constructive criticism, I needed to achieve my goals. I am proud to show them, and the rest of my supportive family, the product of my work.

I also would not have accomplished the culmination of seven years of both undergraduate and graduate studies at Texas A&M University at Galveston without Dr. Jaime Alvarado Bremer, Dr. Susan Knock, and Dr. Luis A. Hurtado. Thank you for your support not only as my committee chair and members but as professors, mentors, and collaborators as I progressed through this research. Had I not been opened up to the opportunities they provided, the professional examples they set, and the inspiring passion for the scientific fields, I would not have come this far in my academic career. Many thanks to Dr. Chris Brown and zoologists at the Dallas Zoo for samples of Caribbean Flamingo and other species, Dr. Miguel A. Mora (TAMU) for Attwater's Prairie Chicken specimens, Dr. Luis A. Hurtado for Greater Roadrunner samples, and Dr. Rodrigo Migoya and personnel of Niños y Crías A.C. for the nonpareil chance to sample Caribbean Flamingos in the Yucatán.

This project was generously supported by the McDaniel Charitable Foundation and the Texas Institute of Oceanography. I'd like to express my gratitude for the aid of the Department of Marine Biology and Office of Graduate Studies as well.

Thanks also go to my co-workers and friends, Brad Smith and Ching-Ping Lu, for taking me under their wing and patiently training me to be a successful and independent researcher in the lab.

NOMENCLATURE

bp	Base Pairs
CHD	Chromosome Helicase ATPase DNA Binding
ddH_2O	Double Deionized Water
DNA	Deoxyribonucleic Acid
Etbr	Ethidium Bromide
F or Fwd	Forward
HRM	High-resolution Melting
HRMA	High-resolution Melting Analysis
ID	Identify, Identification
NGO	Non-government Organization
PCR	Polymerase Chain-reaction
R or Rev	Reverse
RT	Real Time
SA	Short-amplicon
SNP	Single-nucleotide Polymorphism

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1. INTRODUCTION TO AVIAN GENDER DETERMINATION

1.1 *The Importance of Gender Identification in Birds*

Approaches to unambiguously identify (ID) gender of individuals in populations is necessary for proper assessment of demographic parameters used in population dynamics studies, as well as for the characterization of sex-specific migration patterns, comparative behavior, ecology, and evolutionary biology. Accordingly, this information is extremely valuable towards wildlife conservation and management of protected species (Fridolfsson A.K. and H. Ellegren 1999; Donohue, K.C. and A.M. Dufty, Jr. 2006; Balkiz et al. 2007). In birds, this is particularly relevant since roughly half of all avian species lack obvious sexually dimorphic characters (Griffiths et al. 1998) in body size, feather color, or breeding ornamentation (Cerit, H. and K. Avanus 2007). Further, it is often very difficult or impossible to differentiate between males and females outside the breeding season in some species (Donohue, K.C. and A.M. Dufty, Jr. 2006) and is particularly challenging for nestlings and juveniles (Griffiths and Tiwari 1993; Fridolfsson A.K. and H. Ellegren 1999). Gender ID methods are especially useful to help maintain a balance in sex ratio (Studer-Thiersch 1986; Cerit, H. and K. Avanus 2007) of small populations (Griffiths et al. 1998; Childress et al. 2005; Chang et al. 2008; Santamaria et al. 2010) to help prevent declines in the genetically effective population size (Ryman, N. and L. Laikre 1991).

1.2 *Background on Caribbean Flamingo: Subject Species*

Also known as American or Rosy Flamingo, *Phoenicopterus ruber ruber* is distributed primarily throughout the Bahamas, Cuba, Yucatán, Bonaire Island, Venezuela, and Colombia (**Figure 1-1**). Four main breeding sites are known: Great Inagua, Bahamas; Ría Lagartos, Yucatán, Mexico; Bonaire Island, former Netherland Antilles; and James Island (San Salvador or Santiago), Galapagos, Ecuador. Flamingos often make daily flights between breeding and feeding sites. Long-distance migrations include seasonal movements from breeding to wintering sites in Bahamas, Cuba, Dominica and Haiti, and along the Yucatán coast. Caribbean Flamingo from Bonaire winter in Venezuela, Colombia, and Surinam (Richardson, T. and S. Pickering 2005).

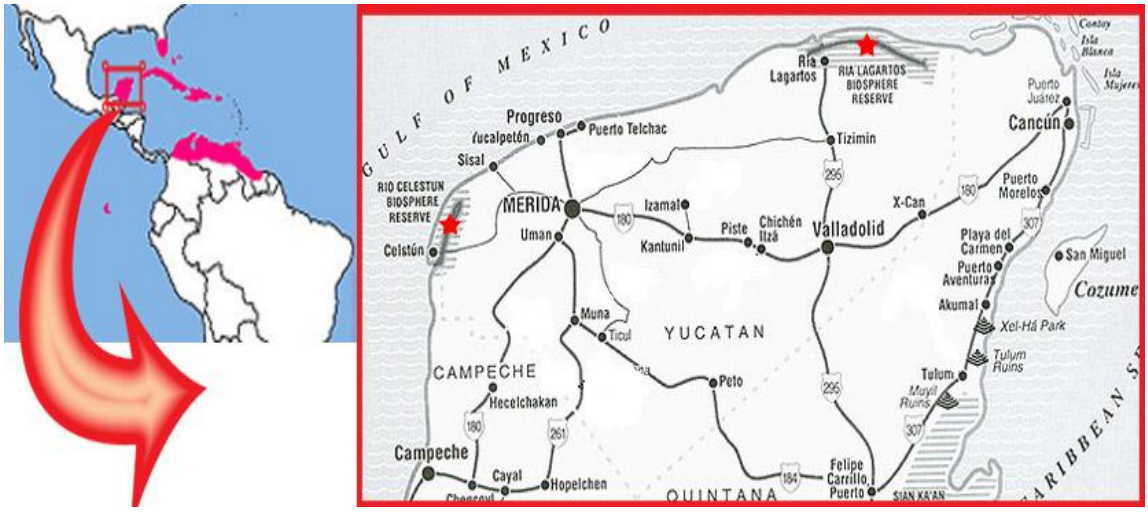

Figure 1-1 Maps of range (in red) of Caribbean Flamingo and the study area in Yucatán, Mexico. Specific study sites at the Biosphere Reserves in Yucatán, Mexico are highlighted (BirdLife International 2012.; MEXonline.com 2012).

Caribbean Flamingo frequent shallow lagoons and mudflats, occasionally preferring man-made solar salt concentration ponds (R. Migoya Pers. Comm.). It is in these bodies of water that they submerge their heads upside-down to filter feed on mollusks and arthropods, skimming from the water column. Diet consists of brine shrimp (*Artemia spp.*), sea snails (*Cerithium lutosum*), clams (*Gemma purpurea*), and brine fly (*Ephydra pupae, E. gracilis*) (Richardson, T. and S. Pickering 2005).

Breeding pairs form dense nesting colonies, often numbering thousands of individuals. Since these birds are highly gregarious, sexual displays ensure synchronized breeding during the most advantageous environmental conditions. All Flamingos (six species) lay a single egg each season and both parents alternate between incubation and feeding. Chicks hatch after 28 and 30 days and begin feeding at two weeks of age, although they continue to be aided by adults until fledging around two and a half months. Chick mortality is primarily attributed to avian predators, although deaths due to territoriality and nest defense are known to occur.

Based exclusively on records of captive individuals, flamingos are considered amongst the longest-lived birds. The oldest Caribbean Flamingo died at age 44 in the Philadelphia Zoo. Estimates of population size for wild flamingos are difficult to obtain since their habitats are often inaccessible and spread across vast geographic regions (Richardson, T. and S. Pickering 2005). Despite the challenges, the International Waterbird Census (IWC) publishes coordinated efforts to estimate populations. The Waterbird Population Estimates established from data from 2002-2006 indicate that of eighteen Phoenicopterid metapopulations, thirteen are either stable or increasing. The

most recent censuses for Caribbean Flamingo conducted in 2005 revealed 490 individuals in Galapagos Islands, 50,000 on the coast of Venezuela, east Columbia, and Dutch Antilles, 40,000 in Yucatán, Mexico, and 167,000-242,000 in Bahamas and Cuba. Greater flamingos in Mauritius are extinct and populations of Lesser Flamingo in Eastern Africa and Andean Flamingo are decreasing (Wetlands International 2006).

Apart from population trends, flamingos are losing the high-quality habitat needed for breeding and feeding. Drastic natural and anthropogenic changes in hydrology can alter ecology of the sensitive coastal niches. Drought, hurricanes, industrial activity, and population growth may alter salinity and in turn reduce prey items, flood nest sites, expose access for predators, and change pH and increase contaminants from run-off among other concerns (Richardson, T. and S. Pickering 2005). Conservation and management techniques can be intertwined with anthropogenic action, as in the case of salt mining operations. Las Coloradas, Yucatán, Mexico is host to a salt mining company (Espino-Barros, R. and G. Baldassarre 1989; Arengo, F. and G.A. Baldassarre 1998). Evaporation ponds are stocked with cultured *Artemia spp.* to control cyanobacterium and algae, a practice that has also been successful in attracting the Caribbean Flamingos to return to nest and feed (C. Brown Pers. Comm.; R. Migoya Pers. Comm.). It is here in the protected Biosphere Reserves that managers and conservationists can monitor the status of the population (Richardson, T. and S. Pickering 2005).

1.3 *General Methods for Gender ID of Birds in the Field*

Several approaches to sex monomorphic species of birds have been used in the field (Cerit, H. and K. Avanus 2007), including vent sexing, which is performed manually by trained specialists who examine the cloaca of hatchlings (one-day old). Chicks are held upside down to detect presence of a rudimentary male sex organ. Experts can correctly ID gender with 95% accuracy but less experienced have a lower success rate (60-70%).

Laparoscopy is a surgical technique executed by veterinary surgeons and provides immediate and precise results about gender. The bird is anesthetized and the instrument is inserted into a small incision in the abdominal wall, at which point, gonadal tissue-type can be examined. Laparoscopy holds the risk of causing inadvertent injury or lead to death, and birds require post-operative intensive care (Cerit, H. and K. Avanus 2007). Accordingly, this method is not applicable in field studies, particularly when dealing with protected species.

Ultrasonography to determine fetal sex is widely used for humans and other mammals. In birds, it has been used for *in situ* visualization of intracloacal structures in raptors successfully confirming gender (Hildebrandt et al. 1995). The availability of hand-held ultrasound devices (e.g. GE© Vscan™) (General Electric Healthcare 2000) offer potential for field research applications but there are no current field studies reporting the use of this technology.

The procedures mentioned above have shortfalls. Vent sexing is very intrusive for sensitive life stages and poses a particular risk for juvenile waterbirds with long legs,

as these may be fractured or harmed when attempting to invert the bird for cloacal inspection (C. Brown, Pers. Comm.). Laparoscopy can be equally damaging or even fatal (Cerit, H. and K. Avanus 2007), especially for small-bodied birds and nestlings (R. Migoya, Pers. Comm.). More importantly, neither approach is practical when studying remote populations (Childress et al. 2005; Donohue, K.C. and A.M. Dufty, Jr. 2006).

An alternative to labor-intensive morphological comparisons and tissue collection is direct observation (Kahn et al. 1998). In some species of birds, it is possible to witness copulation, courtship behaviors, and egg-laying but these events are limited to breeding season in wild populations and require a significant time investment on the part of the observer (Donohue, K.C. and A.M. Dufty, Jr. 2006).

1.4 *Development of Molecular Techniques for Avian Gender Determination*

Current molecular methods of non-ratite avian gender determination target sequence polymorphisms contained in the Chromosome Helicase ATPase DNA Binding (CHD) gene. Ratites (flightless birds like the ostrich) and carinates differ in that the latter have two distinguishable CHD genes from evolutionary divergence of the sex chromosomes (García-Moreno, J. and D.P. Mindell 2000; but see Han et al. 2009). Accordingly, in all bird species except for ratites, females are heterogametic (ZW) and males homogametic (ZZ) (Griffiths et al. 1992). The majority of current methods are centered on scoring intron size differences between CHD-Z and CHD-W genes (**Figure 1-2)** amplified using PCR and separated through gel electrophoresis (Griffiths et al. 1998; Kahn et al. 1998; Fridolfsson A.K. and H. Ellegren 1999). Most studies

characterizing gender in natural populations have utilized primer combination P2 and P8 (Griffiths et al. 1998) or 2550F and 2718R (Fridolfsson A.K. and H. Ellegren 1999), with size polymorphisms scored through 2-3% agarose gels. Occasionally, however, these 'universal' avian gender identification primers are not diagnostic. For instance, differences in intron sizes using primers P2 and P8 are too small (3-8 bp) in certain species to be scored reliably through agarose gels, requiring the higher resolving power of acrylamide gels (Griffiths et al. 1998; Kahn et al. 1998).

An alternative to the use of acrylamide gels to score small fragment size differences is to generate W-specific restriction patterns (Griffiths et al. 1996; Dawson et al. 2001; Boutette et al. 2002; Sacchi et al. 2004) followed by agarose gel electrophoresis. This approach, although reliable, adds an additional step to the gender ID assays. To circumvent the need of restriction enzymes or acrylamide gels, multiplex PCR assays involving CHD-W specific primers have been developed (e.g., Shizuka, D. and B.E. Lyon 2008; Han et al. 2009; Wang et al. 2010). Specifically, the multiplex PCR approach developed by Han et al. (2009) consisted in the inclusion of a third W-specific primer, P0 F (**Figure 1-2**), to be used in combination with primers P2 and P8 (Griffiths et al. 1998). An innovation of this multiplex assay was the use of inosine at the third residue from the 3' end of the W-specific primer that functions to increase the specificity of the reaction. This method described by Han et al. (2009) represents a marked improvement towards increasing the reliability of molecular gender determination assays that utilize agarose gel electrophoresis for scoring.

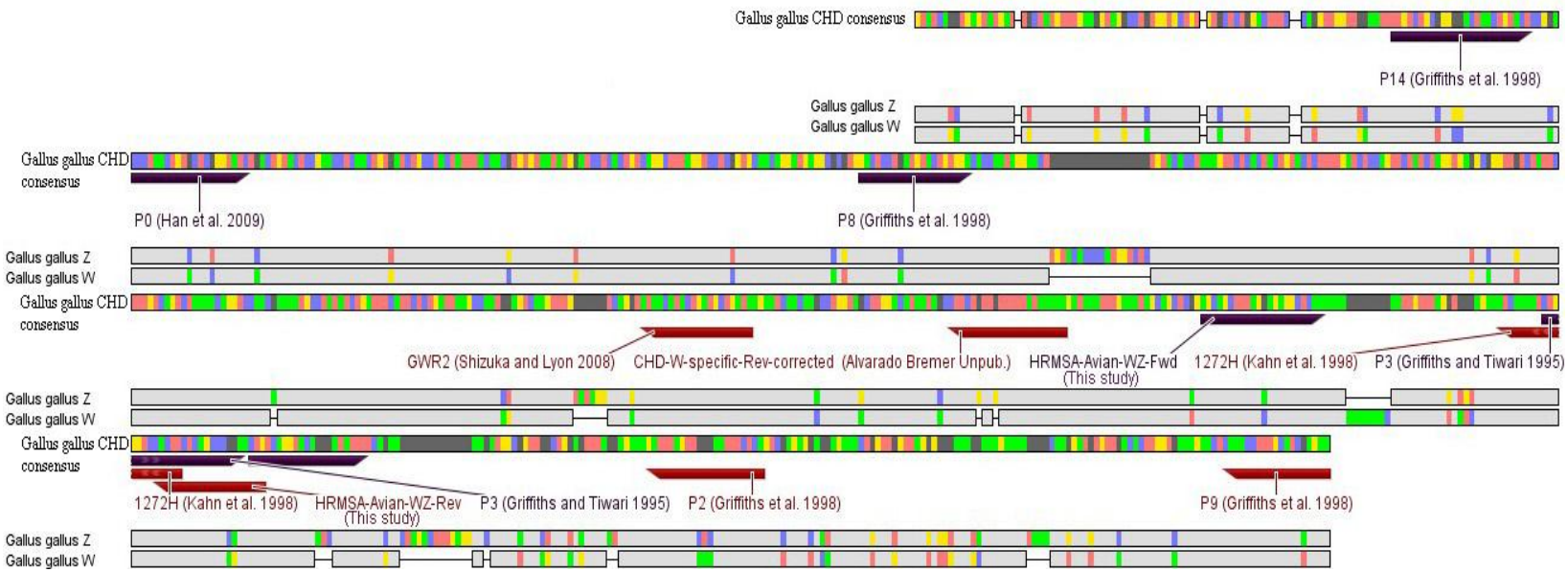

Figure 1-2 PCR primer binding sites along the avian CHD gene (grey) utilized for gender ID. Nucleotides are represented in color along the CHD-W and CHD-Z genes and arrows indicate the direction of forward (5' to 3') and reverse primers (3' to 5') in reference to Chicken (*Gallus gallus*). Therefore, the placement of annotations are approximate and size difference between W and Z amplification products vary from species to species. Note: The positions of primers P3 and P2 are switched in Figure 1 of Ellegren (1996).

Although the intron size differences revealed by primers 2550 F and 2718 R are larger than those produced using primers P2 and P8, Fridolfsson A.K. and H. Ellegren (1999) were not able to identify the gender of three bird species (all Passerines) out of a total of 50. Gender of these three species was identified when two new primer sets were designed.

Additional potential difficulties in CHD-based molecular sexing include the presence of polymorphisms in the Z-gene, as in the case of auklets. Heterogametic males (ZZ'), with different allele sizes, can be erroneously scored as females (Dawson et al. 2001) when using PCR primers P2 and P8. The authors of that study strongly recommend that sexing primers should be initially tested with validated samples in order to identify the primer combination best suited to the study species.

The need to establish highly accurate non-invasive or minimally invasive techniques for gender ID, that are fast, inexpensive, and amendable for high throughput (Griffiths et al. 1998), has only been partially fulfilled. Since these methods involve PCR, the requirement of non-invasiveness or minimal invasiveness is fulfilled because only small amounts of tissue preserved in a variety of ways (e.g., a small drop of dry blood blotted on filter paper) are required as DNA source. When drawing blood is not feasible during fieldwork or in those instances when the procedure may cause damage to delicate specimens, feathers can be plucked instead. The quill tip (calamus) (Santamaria et al. 2010) provides a sufficient source of DNA (Griffiths and Tiwari 1995; Donohue and Dufty, Jr. 2006; Balkiz et al. 2007).

The collection of biological sources of DNA from live birds for the purpose of genotyping should yield quality samples while minimizing discomfort or potential injury to the animal (Cooey 2008). Balkiz et al. (2007) advised use of several small to medium-sized feathers plucked from a region other than the wing or tail to avoid any damage to the bird. In the field, sampling blood and non-primary feathers is carried out for DNA or hormone-level analysis (Cerit, H. and K. Avanus 2007).

All methods described still involve a minimum of three additional steps following DNA isolation: PCR amplification, gel electrophoresis, and photo-documentation for scoring. With each additional step there is an increment in the amount of time, the associated costs, and the chance of introducing errors, while at the same time, the potential for high throughput processing and automation is limited (Alvarado Bremer, Pers. Comm.).

1.4.1 *Melting Curve Analysis for Avian Gender ID Species*

In an effort to reduce some of these limitations, Chang et al. (2008) developed a SYBR®-Green Real Time (RT) PCR combined with melting curve analysis to genotype the intron-size differences of CHD-Z and CHD-W in Formosan Crested Serpent Eagles (*Spilornis cheela hoya*) and in Chinese Bulbuls (*Pycnonotus sinesis*). Unfortunately, the assay was only useful to distinguish the intron-size difference in *S. c. hoya* (13 bp), but not in *P. sinesis* (52 bp), for reasons the authors were unable to track. One possibility could be linked to the low resolution saturating characteristics of SYBR®-Green dye.

Recently, Brubaker et al. (2011) amplified the CHD region of Japanese quail (*Coturnix japonica*) and Eastern Screech-owls (*Megascops asio*) using primers 2550F and 2718R (Fridolfsson and Ellegren 1999). Melting curves for female Japanese quail were homoduplex and males were heteroduplex. For Eastern Screech-owls, males showed two peaks and females showed three, corresponding to multiple melting domains of PCR-amplified products. Later, Morhina et al. (2011) modified their approach using primers P2 R/P8 F and HRMA for these two species. While the melting curves generated were diagnostic, they are all based on targeting intron size differences. Accordingly, preferential amplification of multiple melting domains in fragments of this size may mask diagnostic peaks in other species.

1.5 *Gender Identification of Flamingo Species*

All six Phoenicopterid species lack sexually dimorphic plumage, including at maturity or during breeding season (Richardson, T. and S. Pickering 2005). Studer-Thiersch (1986) first generalized gender ID for the members of genus *Phoenicopterus* based on records of tarsus length for adult Greater Flamingo, Caribbean Flamingo, and Chilean Flamingo. Individually sexed Greater Flamingo at a zoo was achieved via behavioral observations or laparoscopy. Tarsus length was only applicable to 'small' and 'large' adult Greater Flamingo and Chilean Flamingo whereas overlapping measurements for 'medium-sized' birds misidentified sex. Any variability in tarsus length after six to ten weeks of age is attributed to age and not gender. By eight or nine months, sexes are indistinguishable (Studer-Thiersch 1986).

Bertault et al. (1999) encountered a similar scenario with Greater Flamingo (*Phoenicopterus ruber roseus*) to that described by Griffiths et al. (1998) for Tawny Owl, where differences between CHD-Z and CHD-W genes are very small. Amplified products using primers P2 R and P8 F of 133 Greater Flamingo chicks from Camargue, France were digested with *Mbo*II and run in a 3% agarose gels to distinguish gender. In addition, Bertault et al. (1999) successfully applied the same protocol to determine gender of Chilean Flamingo (*Phoenicopterus chilensis*). Tomasulo et al. (2002) replicated this assay and found that it is possible to ID gender in Chilean Flamingo without using digestion with *Mbo*II.

Childress et al. (2005) correlated differences in body measurements with sex of Lesser Flamingo (*Phoenicopterus minor*) in an attempt to ascertain the gender from specimens captured in the field without having to rely on molecular methods. Body mass, head-and-bill length, culmen length, flattened wing length, and tarsus length was obtained from 154 Lesser Flamingos corresponding to three age groups: adults less than three years of age, sub-adults two to three years old, and first-year juveniles. Gender ID was validated using DNA extraction from blood samples and amplified using primers P2/P8 (Griffiths et al. 1998) run in 2% agarose gels.

Results from that study showed that on average, the females of Lesser Flamingo are smaller than males but there is considerable overlap of size measurements. Mass alone was the least reliable proxy for sexing Lesser Flamingo, correctly predicting it in 79% of cases overall and in 65% of first-year juvenile birds (Childress et al. 2005). Further, since large females and small males occur, using weight to ID gender can be

deceptive. However, head-and-bill length correctly predicted the sex of Lesser Flamingo from all three age classes with 93% accuracy. Conversely, total tarsus length correctly predicted sex with 94% accuracy in adults but only 61% in juveniles and 73% in immature sub-adults.

Multivariate discriminant functions specific to each age class were developed and applied to 19 captive adult Lesser Flamingos, positively identifying gender of all. These morphometric relationships concerning this species would be greatly useful for researchers in the field but the authors noted some limitations. The best combination to ID gender was head-and bill length with tarsus length, increasing positive gender ID to 98%, but only for adults. No improvement was gained by adding tarsus length for immature or juvenile birds. It is also impractical to monitor isolated populations (Donohue, K.C. and A.M. Dufty, Jr. 2006) like the inhospitable breeding sites of Lesser Flamingo (Childress et al. 2005). Similar studies have not been applied to Caribbean Flamingo.

Likewise, the average adult male Greater Flamingo (*Phoenicopterus ruber roseus*) weighs more than an adult female and gender can be determined with little error, though the same is not true for chicks (Balkiz et al. 2007). DNA extracted from calamus tissue was amplified with 2550 F and 2718 R (Fridolfsson A.K. and H. Ellegren 1999), yielding a single band present in a 1.8% agarose gel for some females. Primer competition between amplifications of CHD-W and CHD-Z in combination with poor DNA quality was suggested as the cause preventing amplification of the CHD-W gene in some females (Balkiz et al. 2007).

Preliminary amplifications were generated using several primer combinations and feather samples from Caribbean Flamingo specimens (n = 17) provided by the Dallas Zoo (**Table 1-1**). Several primer combinations were in used in attempt to ID gender of all (**Table 1-2**).

Table 1-1 Specimen list for feather samples from Caribbean Flamingo (*P. ruber ruber*). Samples were provided by zoologists at the Dallas Zoo. Specimen ID refers to the individual's International Species Information System (ISIS) code. Highlighted in pink are two specimens whose gender was misidentified by the zoo.

	Specimen ID	Gender (Given)	HRM Date (MM/DD/2009)	Gentoype (HRM)	Gender (HRM)	Direct Sequencing	Remarks
1	000D92	♂	04/23	Homoduplex	♂	n/a	Melting Curve Atypical
2	03E897	♂	04/23	Homoduplex	♂	n/a	n/a
3	03E899	♂	04/23	Homoduplex	♂	n/a	n/a
4	03E901	♂	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW
5	03E903	♂	04/23	Homoduplex	♂	n/a	Melting Curve Atypical
6	03E906	♂	04/23	Homoduplex	♂	n/a	n/a
7	03E908	♂	04/23	Homoduplex	♂	n/a	n/a
8	03E912	♂	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW
9	03E913	♂	04/23	Homoduplex	♂	n/a	n/a
10	03E914	♂	04/23	Homoduplex	♂	P2-P14	Confirmed ZZ
11	03E898	♀	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW
12	03E900	♀	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW
13	03E902	♀	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW
14	03E907	♀	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW
15	03E910	♀	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW
16	03E911	♀	04/23	Heteroduplex	♀	n/a	n/a
17	03E918	♀	04/23	Heteroduplex	♀	P2-P14	Confirmed ZW

Similar to certain birds (Griffiths et al. 1998; Bertault et al. 1999; Fridolfsson and Ellegren 1999; Chang et al. 2008; Han et al. 2009), the banding patterns of P2 R/P8 F PCR products were not diagnostic for gender of Caribbean Flamingo. Variations in

intron length between W and Z fragments were too small to be visualized in a 2.5% EtBr-stained gel (**Figure 1-3**).

Table 1-2 Primer combinations attempted to ID gender of 17 Caribbean Flamingo from the Dallas Zoo.

	Forward Primer	Reverse Primer	Multiplex Primer (if applicable)	Genotyping Method	Remarks
1	P8 (Griffiths et al. 1998)	P2 (Griffiths et al. 1998)		2.5% Agarose-gel; EtBr stained	No amp. of W in females; Sequenced directly (~362bp); [MgCl2] at 2mM - No amps.; Increased [MgCl2] to 3mM
2	2550F (Fridolffson and Ellegren 1999)	2718R (Fridolffson and Ellegren 1999)		2.5% Agarose-gel; EtBr stained	Touch-down PCR to optimize temp.; No amps.
3	CHD-Flamingo (Alvarado Bremer Unpub.)	P2 (Griffiths et al. 1998)		2.5% Agarose-gel; EtBr stained	Flamingo-specific primer from direct sequencing Z and W in some - plugs of upper band (W) sequenced directly
4	P14 (Griffiths et al. 1998)	P2 (Griffiths et al. 1998)		2.5% Agarose-gel; EtBr stained	Single band (~500bp)
5	P8 (Griffiths et al. 1998)	CHD-W (Alvarado Bremer Unpub.)		2.0% Agarose-gel; EtBr stained	CHD-W specific primer from direct sequencing Over-amplification; 2 bands not differentiable
6	P14 (Griffiths et al. 1998)	CHD-W (Alvarado Bremer Unpub.)		2.0% Agarose-gel; EtBr stained	Z and W in all; Strong amps - plugs of upper band (W) sequenced directly
7	P14 (Griffiths et al. 1998)	P9 (Griffiths et al. 1998)		1.8% Agarose-gel; EtBr stained	Direct sequencing to identify unknown product
8	CHD-WZ-F-Avian Universal	CHD-WZ-R-Avian Universal		2.5% Agarose-gel; EtBr stained	2 bands not differentiable
9	CHD-WZ-F-Avian Universal	CHD-WZ-R-Avian Universal	CHD-W-specific nt 215	2.5% Agarose-gel; EtBr stained	Multiple bands; No pattern
10	CHD-WZ-F-Avian Universal	CHD-WZ-R-Avian Universal	Avian-SexDeterm-probe	HRMA	Melt. Curves of Z and W not differentiable
11	P8 (Griffiths et al. 1998)	P2 (Griffiths et al. 1998)		HRMA	Primer dimer suspected in melting Confirmed dimer in 2.5% Agarose-gel - EtBr stained
12	P14 (Griffiths et al. 1998)	P2 (Griffiths et al. 1998)		HRMA	Multiple melt. Curves
13	CHD-WZ-F-Avian Universal	CHD-WZ-R-Avian Universal		HRMA	Increased # of cycles to 55; Multiple melt. Curves
14	HRMSA-Avian-WZ-F	HRMSA-Avian-WZ-R		HRMA	Melt. Curves diagnostic; Increased # cycles from 45 to 60

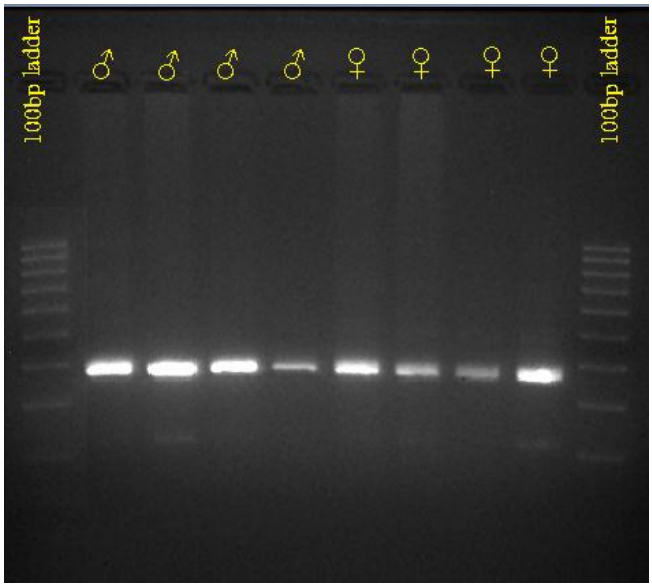

Figure 1-3 Amplification of Caribbean Flamingo using P2 R and P8 F. Amplicons were visualized in a 2.5% Ethidium Bromide (Etbr)-stained agarose gel. Four male (♂) and four female (♀) Caribbean Flamingo are compared. Females (WZ) were expected to display two bands differing in size.

2. DNA MELTING ANALYSIS FOR GENOTYPING

2.1 *High-resolution Melting: A New Molecular Genotyping Method*

High-resolution melting analysis (HRMA) has become a powerful tool for the rapid characterization of genetic variations - detecting Single-nucleotide Polymorphisms (SNPs), indels (insertion-deletion events), and methylations (Wittwer 2009). HRMA is fast, highly sensitive, inexpensive, and has potential for high throughput. It is a closed-tube method that requires minimal amounts of tissue for DNA extraction.

Sample analysis of PCR-amplified DNA segments differing by one mutation melt at different temperatures, and these can be visualized as changes in fluorescence. Further, when using the generic dye LC-Green, heterozygous genotypes can be easily distinguished by their characteristic multimodal heteroduplex curves that differ from the unimodal curves of homozygous (homoduplex) genotypes (Wittwer 2009).

Until recently, HRMA was primarily used in clinical studies to diagnose informative genomic mutations like SNPs. Smith et al. (2009) demonstrated its potential as a rapid and highly sensitive tool to study interspecific genetic variability in wild populations.

2.2 *Applicability of HRMA for Gender Identification of Caribbean Flamingo*

We developed HRMA for gender ID of Caribbean Flamingo in particular, with applications to other birds, including seven species of waterbirds, Greater Roadrunner, and Attwater's Prairie Chicken based upon SNPs in a short segment (81 bp long)

without relying on intron size differences. The objective of this study was to first verify if HRMA could be used to identify the gender of Caribbean Flamingo using a single primer set designed for a highly-conserved segment of the ATPase DNA Binding exon of the avian CHD-Z and CHD-W genes.

2.2.1 *Materials and Methods for DNA Extraction*

Extraction of DNA followed the hot sodium hydroxide and tris, or "HotSHOT" protocol described by Balkiz et al. (2007) and Truett et al. (2000) as a means for a quick and inorganic, yet high-quality DNA isolation method.

When using blood blotted onto filter paper, a small piece of paper, approximately $1mm^2$, was clipped from each sample using sterilized scissors and tweezers. When only feathers were available, a $1mm^2$ portion of the calamus was clipped. Samples were transferred into 1.5 ml Eppendorf tubes containing 40 microliters (µl) sodium hydroxide (NaOH) 0.2N then vortexed gently for 10 seconds. The samples were then placed on a VWR Select Heatblock at 75°C for 20 minutes. After vortexing samples for 10 seconds, they were placed on ice to cool to about 4°C. Finally, 40 µl Tris-HCl pH 8 and 120 µl of double deionized water (ddH_2O) were added to the samples which were then centrifuged (Fisher Scientific accuSpin™) at 13,000rpm for one minute. The DNA extract was stored at -20°C after this point. Using 1 µl of the supernatant was used as DNA template in PCR.

2.2.2 *Materials and Methods for DNA Amplification and Sequencing*

To verify the results obtained from HRMA, the CHD-W and CHD-Z alleles were PCR amplified and sequenced. Reactions were prepared in 12.5 µl volumes containing: 8.525 µl ddH_2O, 1.25 µl 10x buffer, 0.375 µl 50 mM $MgCl_2$, 0.25 µl dNTPs, 0.5 µl of each primer - P0 F (Han et al. 2009), P2 R, and P14 (Griffiths et al. 1998), 0.1 µl Platinum® *Taq* polymerase (Invitrogen), and 1 µl of isolated DNA template. To only target the Z chromosome, samples from males were amplified with primers P2 R and P14 F. To only amplify the W chromosome of females, PCR reactions included primers P2 R and P0 F. Sequences were submitted to Genbank (see **Appendix Table B-1**).

DNA amplification was carried out in an Eppendorf Mastercycler® Gradient thermal cycler. An initial denaturing step of 2 minutes at 94°C was followed by 35 cycles of strand denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. The final extension was at 72°C for 3 minutes. 5 µl of each PCR product was then loaded into a 2.5% agarose gel pre-stained with 0.1 µg/ml Ethidium Bromide (Etbr), allowed to run at 100 mV for 50 minutes, and viewed through an ultraviolet transilluminator to determine the quality of the amplifications.

In preparation for sequencing in the ABI 3130 genetic analyzer (Applied Biosystems, Foster City, California), excess primers and dNTPs were removed from PCR products by the addition of 2 µl of ExoSAP-IT™ (USB Corporation, Cleveland, Ohio) following the manufacturer's recommendations. The BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California) was used

in the cycle sequencing reaction. This reaction involved combining 1 µl of BigDye™, 2 µl 5x dilution buffer, 5 µl of ddH_2O, and 1 µl of clean PCR product, with 1 µl of HRMSA-Avian-WZ-Fwd and HRMSA-Avian-WZ-Rev primers in new 0.2 ml tubes. After a pulse in the centrifuge, the samples were reloaded into the thermal cycler for cycle sequencing, which consisted of 22 cycles of the following profile: 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes and 15 seconds. Lastly, the BigDye® XTerminator Purification Kit was employed to remove unincorporated BigDye® terminators to improve the quality of sequence reads. 11.25 µl of warm SAM™ Solution and 2.5 µl of XTerminator™ Solution were added to each sample. The samples were then vortexed for 30 minutes and centrifuged for one minute at 13,000 rpm. The size of the fragment sequenced was ≈514 bp.

The resulting DNA sequences were aligned using ClustalW and edited using the sequence alignment editor Geneious Pro (Biomatters Development Team 2010). This software was also used to align the sequences of other avian species and to visualize the fit of the HRMSA primer set.

Polymerase chain reactions were prepared in 10 µl of final volume in LightCycler® capillaries (Roche). The reaction contained 1.5 µl ddH_2O, 1 µl 10x BSA (2.5mg/ml), 0.5 µl LCGreen®Plus, 0.5 µl of each primer (HRMSA-Avian-WZ-Fwd and HRMSA-Avian-WZ-Rev), 5 µl 2x EconoTaq® PLUS polymerase (Lucigen), and 1 µl of isolated DNA template. 15 µl of mineral oil was also added to each capillary.

DNA amplification was carried out in a Rapid Cycler2® (Idaho Technology Inc., Salt Lake City, UT). An initial denaturing step of 1 minute at 94°C was followed by 70

cycles of strand denaturation at 94°C for 0 seconds, primer annealing at 50°C for 0 seconds, and extension at 72°C for 12 seconds. Negative controls (no DNA) were run with every reaction and standard precautions were taken to screen for potential contamination.

Melting analyses of the PCR amplifications were carried in an HR-1 Instrument (Idaho Technology Inc., Salt Lake City, UT). The melting protocol entailed a ramp rate of 0.3°C/sec, data acquisition beginning at 76°C, a final temperature of 82°C, and cooling to 59°C. Derivative plots of fluorescence against melting temperatures were generated using HR-1 Melting Analysis tools.

2.3 *Results*

Contiguous DNA sequences generated from PCR-amplified segments using P2 R and P14 F were aligned and compared to an existing entry of the CHD-Z of *Phoenicopterus ruber ruber* in GenBank (Accession AF440751). A short-amplicon (SA) HRMA assay (**Figure 2-1A**) utilizing a single primer set was designed after aligning the CHD-Z and CHD-W sequences of Caribbean Flamingo. Sequencing revealed that the difference in intron-size is only 8 bp long (**Figure 2-1B**).

Figure 2-1 Sequence of CHD-Z and CHD-W amplified segments of Caribbean Flamingo (*P. ruber ruber*). Differences (consensus) between the two alleles and the placement of **A** HRM short-amplicon (SA) Avian-WZ primer set and **B** the placement of universal avian gender ID primers P0 F, P8 F, and P2 R are shown. Primer P14 is not shown.

SNPs within orthologous portions of the CHD-Z and CHD-W exon in sequences from 17 Caribbean Flamingo supplied by the Dallas Zoo were successfully amplified. Fluorescence derivative plots were generated and unimodal melting curves correspond to homozygous males (ZZ) whereas those of heterozygous females (WZ) are bimodal (**Figure 2-2**).

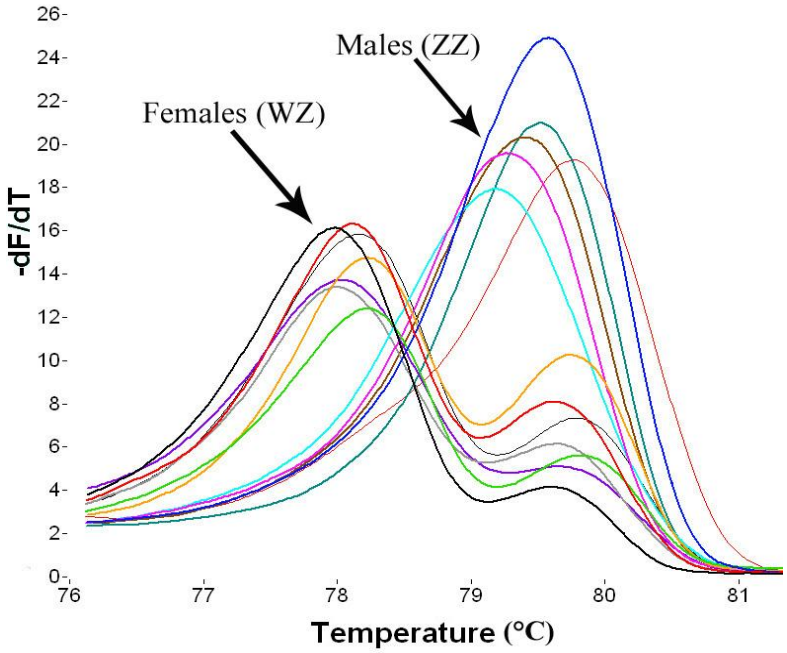

Figure 2-2 Avian gender ID HRMA of Caribbean Flamingo (*P. ruber ruber*). The fluorescence derivative plot unimodal melting curves of males (ZZ) and females bimodal melting curves of females (WZ) are shown. Each curve represents one individual.

Melting ranged from 76-81°C. Females (WZ) produce earlier peaks around 78°C and smaller peaks around 79.5°C. Males (ZZ) have only a strong unimodal peak around 79.5°C.

Of the 17 Caribbean Flamingo analyzed, two individuals originally identified as males by the Dallas Zoo produced heteroduplex curves, indicating they were females (see **Table 1-1**). Resequencing of the CHD gene using primers P0 F/P2 R confirmed these two individuals as females.

3. A UNIVERSAL MOLECULAR METHOD FOR AVIAN GENDER IDENTIFICATION

3.1 *Applicability of HRMSA to Other Avian Species*

Dr. Luis A. Hurtado from the Department of Wildlife and Fisheries Sciences at Texas A&M University (TAMU) provided a sample (n = 71) of Greater Roadrunner (*Geococcyx californianus*), for which blood from specimens are listed in **Table 3-1**. The gender of nine specimens had been determined via necroscopic exam and the rest behaviorally (L.A. Hurtado, Pers. Comm.). Amplifications using P2 R / P14 F and P0 F / P2 R (see **Figure 1-2**) were conducted to respectively target the CHD-Z and CHD-W genes used for sequencing and also as positive controls of results obtained with HRMA.

Of the 71 Greater Roadrunner, five individuals (highlighted in pink in **Table 3-1**) produced melting curves not in agreement to the gender originally identified by Dr. Luis A. Hurtado (TAMU). Multiplex PCR (Han et al. 2009) corroborated results of HRMA.

Table 3-1 Specimen list for blood samples from Greater Roadrunner (*Geococcyx californianus*). Samples were provided by Dr. Luis A. Hurtado (Department of Wildlife and Fisheries Sciences (TAMU)). Highlighted in pink are specimens whose gender was misidentified and symbols (*) indicate special remarks.

	Specimen ID	Gender (Given)	Luis A. Hurtado (2009)	Capillary # (HRM)	HRM Date (MM/DD/2009)	Gentoype (HRM)	Gender (HRM)	Multiplex PCR (Han *et al.* 2009)	Remarks
1	123	n/a	♀	01	12/02	Heteroduplex	♀	n/a	
2	190	n/a	♀	02	12/02	Homoduplex	♂	♂	Very low homoduplex curve
3	1013-B	♀	♀	03	12/02	Heteroduplex	♀	n/a	
4	1353-B	♀	♀	04	12/02	Heteroduplex	♀	♀	
5	4N	n/a	♂	88	11/20	Homoduplex	♂	n/a	
6	F1013	n/a	♀	91	12/04	Heteroduplex	♀	n/a	
7	F1353	n/a	♀	90	12/04	Heteroduplex	♀	n/a	
8	JRR-A	n/a	♀	92	12/05	Heteroduplex	♀	n/a	
9	JRR-B	n/a	♂	93	12/06	Heteroduplex	♀	♀	
10	JRR-C	n/a	♂	94	12/07	Homoduplex	♂	n/a	
11	JRR-D	n/a	♂	95	12/08	Homoduplex	♂	n/a	
12	JRR-E	n/a	♂	96	12/09	Homoduplex	♂	n/a	
13	JRR-F	n/a	♂	13	12/10	Homoduplex	♂	n/a	
14	JRR-G	n/a	♀	14	12/11	Heteroduplex*	♀*	n/a	Curve atypical
15	JRR-G-B	n/a	♀	15	12/12	Heteroduplex*	♀*	n/a	Curve atypical
16	JRR-H	n/a	♂	16	12/13	Homoduplex	♂	n/a	Low melt temp Z'-allele
17	JRR-I	n/a	♀	17	12/14	Heteroduplex*	♀*	n/a	Curve atypical
18	JRR-J	n/a	♀	87	11/20	Heteroduplex	♀	n/a	
19	JRR-K	n/a	♀	18	12/04	Heteroduplex*	♀*	n/a	Curve atypical
20	JRR-L	n/a	♂	19	12/05	Homoduplex	♂	n/a	
21	M1	♂	♂	05	12/02	Homoduplex	♂	♂	
22	M1B	♂	♂	06	12/02	Homoduplex	♂	♂	
23	M2B	♂	♂	07	12/02	Homoduplex	♂	n/a	
24	M3	♂	♂	08	12/02	Homoduplex	♂	n/a	
25	M3B	♂	♂	09	12/02	Homoduplex	♂	n/a	
26	M4	♂	♂	10	12/02	Homoduplex	♂	n/a	
27	M4B	♂	♂	11	12/02	Homoduplex	♂	n/a	
28	RR1-2008	n/a	♂	19	12/04	Homoduplex	♂	n/a	
29	RR2-2008	n/a	♀	21	10/21; 12/04	Heteroduplex*	♂; ♀(rescored)	♀	Curve atypical
30	RR3-2008	n/a	♂	22	12/04	Homoduplex	♂	n/a	
31	RR4-2008	n/a	♂	23	12/04	Homoduplex	♂	n/a	
32	RR5-2008	n/a	♂	24	12/04	Homoduplex	♂	n/a	
33	RR6-2008	n/a	♂	15	12/10	Homoduplex	♂	n/a	
34	RR7-2008	n/a	♀	16	12/10	Heteroduplex	♀	n/a	
35	RR8-2008	n/a	♀	17	12/10	Homoduplex	♂	♂	
36	RR9-2008	n/a	♀	18	12/10	Heteroduplex	♀	n/a	
37	RR10-2008	n/a	♀	19	12/10	Heteroduplex	♀	n/a	
38	RR11-2008	n/a	♂	20	12/10	Homoduplex	♂	n/a	
39	RR12-2008	n/a	♂	21	12/10	Homoduplex	♂	n/a	
40	RR13-2008	n/a	♂	22	12/10	Heteroduplex*	♂*	n/a	Apparently heterozygote for ZZ'
41	RR14-2008	n/a	♀	23	12/10	Heteroduplex	♀	n/a	
42	RR15-2008	n/a	♀	24	12/10	Heteroduplex	♀	n/a	
43	RR16-2008	n/a	♀	25	12/10	Heteroduplex	♀	n/a	

Table 3-1 continued

	Specimen ID	Gender (Given)	Luis A. Hurtado (2009)	Capillary # (HRM)	HRM Date (MM/DD/2009)	Gentoype (HRM)	Gender (HRM)	Han *et al.* (2009) (Multiplex PCR)	Remarks
44	RR17-2008	n/a	♀	26	12/10	Heteroduplex	♀	n/a	
45	RR18-2008	n/a	♀	85	12/10	Heteroduplex	♀	n/a	
46	RR19-2008	n/a	♀	27	12/10	Heteroduplex	♀	n/a	
47	RR1-2009	n/a	♂	28	12/10	Heteroduplex	♀	♀	
48	RR2-2009	n/a	♂	29	12/10	Homoduplex	♂	n/a	Low melt temp Z'-allele
49	RR3-2009	n/a	♀	30	12/10	Heteroduplex	♀	n/a	
50	RR4-2009	n/a	♀	31	12/10	Heteroduplex	♀	n/a	
51	RR5-2009	n/a	♀	56	12/10	Heteroduplex	♀	n/a	
52	RR6-2009	n/a	♂	57	12/09	Homoduplex	♂	n/a	
53	RR7-2009	n/a	♂	58	12/10	Homoduplex	♂	n/a	
54	RR8-2009	n/a	♂	44	12/14	Homoduplex	♂	♂	
55	RR9-2009	n/a	♂	45	12/14	Homoduplex	♂	n/a	
56	RR10-2009	n/a	♂	46	12/14	Homoduplex	♂	n/a	
57	RR11-2009	n/a	♂	47	12/14	Homoduplex	♂	n/a	
58	RR12-2009	n/a	♂	48	12/14	Homoduplex	♂	n/a	
59	RR13-2009	n/a	♀	49	12/14	Heteroduplex	♀	n/a	
60	RR14-2009	n/a	♀	50	12/14	Heteroduplex	♀	n/a	
61	RR15-2009	n/a	♂	51	12/14	Homoduplex	♂	n/a	
62	RR16-2009	n/a	♂	52	12/14	Homoduplex	♂	n/a	
63	RR17-2009	n/a	♀	53	12/14	Heteroduplex	♀	n/a	
64	RR18-2009	n/a	♀	54	12/14; 12/14	Heteroduplex*	♂*; ♀ (rescored)	♀	High-melting heteroduplex curve. ZZ" Male?
65	RR19-2009	n/a	♀	55	12/14	Heteroduplex	♀	n/a	
66	RR20-2009	n/a	♀	56	12/14	Heteroduplex	♀	n/a	
67	RR21-2009	n/a	♀	57	12/14	Heteroduplex	♀	n/a	
68	RR22-2009	n/a	♀	58	12/14	Heteroduplex	♀	n/a	
69	RR23-2009	n/a	♀	86	12/14	Heteroduplex	♀	n/a	
70	RR24-2009	n/a	♂	59	12/14	Heteroduplex	♀	♀	
71	RR25-2009	n/a	♀	60	12/14	Heteroduplex	♀	n/a	

Furthermore, the Dallas Zoo provided blood samples for an additional seven species of waterbirds: Lesser Flamingo (*P. minor*), Saddle-billed Stork (*Ephippiorhynchus senegalensis*), Scarlet Ibis (*Eudocimus ruber*), White-bellied Stork (*Ciconia abdimii*), Roseate Spoonbill (*Platalea ajaja*), Chilean Flamingo (*P. chilensis*), and Marabou Stork (*Leptoptilos crumeniferus*) (**Table 3-2**).

Dried blood on filter paper from three males and three females of each species were provided except for Marabou Stork, for which only three males were provided.

HRMA was suitable to score SNPs in six of the eight aforementioned species (**Figure 3-1**).

Table 3-2 Specimen list for blood samples of seven waterbird species provided by the Dallas Zoo.

	Species	Common Name	Specimen ID	Gender (Given)	Multiplex Date (MM/DD/2010)	Multiplex PCR (Han *et al.* 2009)	HRM Date(s) (MM/DD/2010)	Genotype (HRM)	Gender (HRM)	Direct Sequencing	Remarks
1	*Ephippiorhynchus senegalensis*	Saddle-billed Stork	SB1F	♀	10/21	♀	10/21	Heteroduplex	♀	P2-P0	Confirmed ZW
2	*Ephippiorhynchus senegalensis*	Saddle-billed Stork	SB2F	♀	02/12	♀	02/17	Heteroduplex	♀	P2-P0	Confirmed ZW
3	*Ephippiorhynchus senegalensis*	Saddle-billed Stork	SB3F	♀	10/21	♀	02/11	Heteroduplex	♀	n/a	n/a
4	*Ephippiorhynchus senegalensis*	Saddle-billed Stork	SB1M	♂	10/21	♂	10/21	Homoduplex	♂	P2-P14	Confirmed ZZ
5	*Ephippiorhynchus senegalensis*	Saddle-billed Stork	SB2M	♂	02/12	♂	02/17	Homoduplex	♂	P2-P14	Confirmed ZZ
6	*Ephippiorhynchus senegalensis*	Saddle-billed Stork	SB3M	♂	10/21	♂	02/11	Homoduplex	♂	n/a	n/a
7	*Eudocimus ruber*	Scarlet Ibis	SI1F	♀	n/a	n/a	10/21	Heteroduplex	♀	n/a	n/a
8	*Eudocimus ruber*	Scarlet Ibis	SI2F	♀	10/21	♀	02/11	Heteroduplex	♀	n/a	n/a
9	*Eudocimus ruber*	Scarlet Ibis	SI3F	♀	02/12	♂	10/21	Heteroduplex	♀	P0-P2	Confirmed ZW
10	*Eudocimus ruber*	Scarlet Ibis	SI1M	♂	10/21	♂	02/17	Heteroduplex	♀	P2-P14	Confirmed ZZ
11	*Eudocimus ruber*	Scarlet Ibis	SI2M	♂	02/12	♂	02/17	Homoduplex	♂	P2-P14	Confirmed ZZ
12	*Eudocimus ruber*	Scarlet Ibis	SI3M	♂	10/21	♂	02/11	Homoduplex	♂	n/a	n/a
13	*Leptoptilos crumenifer*	Marabou Stork	MS1M	♂	02/12	♂	02/11	Homoduplex	♂	n/a	n/a
14	*Leptoptilos crumenifer*	Marabou Stork	MS2M	♂	10/21	♂	10/21	Homoduplex	♂	n/a	n/a
15	*Leptoptilos crumenifer*	Marabou Stork	MS3M	♂	10/21	♂	02/11	Homoduplex	♂	n/a	n/a
16	*Ciconia abdimii*	White-bellied Stork	WB1F	♀	10/21	♀	10/18	Heteroduplex	♀	P2-P0	Confirmed ZW
17	*Ciconia abdimii*	White-bellied Stork	WB2F	♀	02/12	♂	02/17; 10/18	Homoduplex	♂	n/a	n/a
18	*Ciconia abdimii*	White-bellied Stork	WB3F	♀	02/12	♀	02/11; 10/18	Heteroduplex	♀	n/a	n/a
19	*Ciconia abdimii*	White-bellied Stork	WB1M	♂	02/12	♀	10/18	Heteroduplex	♀	n/a	n/a
20	*Ciconia abdimii*	White-bellied Stork	WB2M	♂	10/21	♂	02/11; 10/18	Homoduplex	♂	P2-P14	Confirmed ZZ
21	*Ciconia abdimii*	White-bellied Stork	WB3M	♂	02/12	♂	02/17; 10/18	Homoduplex	♂	P2-P14	Confirmed ZZ
22	*Phoenicopterus chilensis*	Chilean Flamingo	CF1F	♀	02/12	♂	10/18	Homoduplex	♂	n/a	n/a
23	*Phoenicopterus chilensis*	Chilean Flamingo	CF2F	♀	02/12	♀	2/17; 10/18	Homoduplex	♂	P2-P0	Confirmed ZW
24	*Phoenicopterus chilensis*	Chilean Flamingo	CF3F	♀	02/12	♀	10/18	Homoduplex	♂	n/a	n/a
25	*Phoenicopterus chilensis*	Chilean Flamingo	CF1M	♂	02/12	♂	10/18	Homoduplex	♂	P2-P14	Confirmed ZZ
26	*Phoenicopterus chilensis*	Chilean Flamingo	CF2M	♂	10/25	♂	2/11; 10/18	Homoduplex	♂	P2-P14	Confirmed ZZ
27	*Phoenicopterus chilensis*	Chilean Flamingo	CF3M	♂	02/12	♂	02/17	Homoduplex	♂	n/a	n/a
28	*Platalea ajaja*	Roseate Spoonbill	RS1F	♀	02/12	♀	02/17	Heteroduplex	♀	P2-P0	Confirmed ZW
29	*Platalea ajaja*	Roseate Spoonbill	RS2F	♀	10/25	♀	02/17	Heteroduplex	♀	P2-P0	Confirmed ZW
30	*Platalea ajaja*	Roseate Spoonbill	RS3F	♀	02/12	♀	02/11	Heteroduplex	♀	n/a	n/a
31	*Platalea ajaja*	Roseate Spoonbill	RS1M	♂	10/25	♂	10/21	Homoduplex	♂	P2-P14	Confirmed ZZ
32	*Platalea ajaja*	Roseate Spoonbill	RS2M	♂	10/25	♂	02/11	Homoduplex	♂	n/a	n/a
33	*Platalea ajaja*	Roseate Spoonbill	RS3M	♂	10/25	♂	02/17	Homoduplex	♂	n/a	n/a
34	*Phoenicopterus minor*	Lesser Flamingo	LF1F	♀	10/25	♀	10/21	Heteroduplex	♀	P2-P0	Confirmed ZW
35	*Phoenicopterus minor*	Lesser Flamingo	LF2F	♀	02/12	♀	02/17	Heteroduplex	♀	P2-P0	Confirmed ZW
36	*Phoenicopterus minor*	Lesser Flamingo	LF3F	♀	02/12	♀	02/11	Heteroduplex	♀	n/a	n/a
37	*Phoenicopterus minor*	Lesser Flamingo	LF1M	♂	10/25	♂	02/11	Homoduplex	♂	P2-P14	Confirmed ZZ
38	*Phoenicopterus minor*	Lesser Flamingo	LF2M	♂	10/25	♂	10/21	Heteroduplex	♂	n/a	n/a

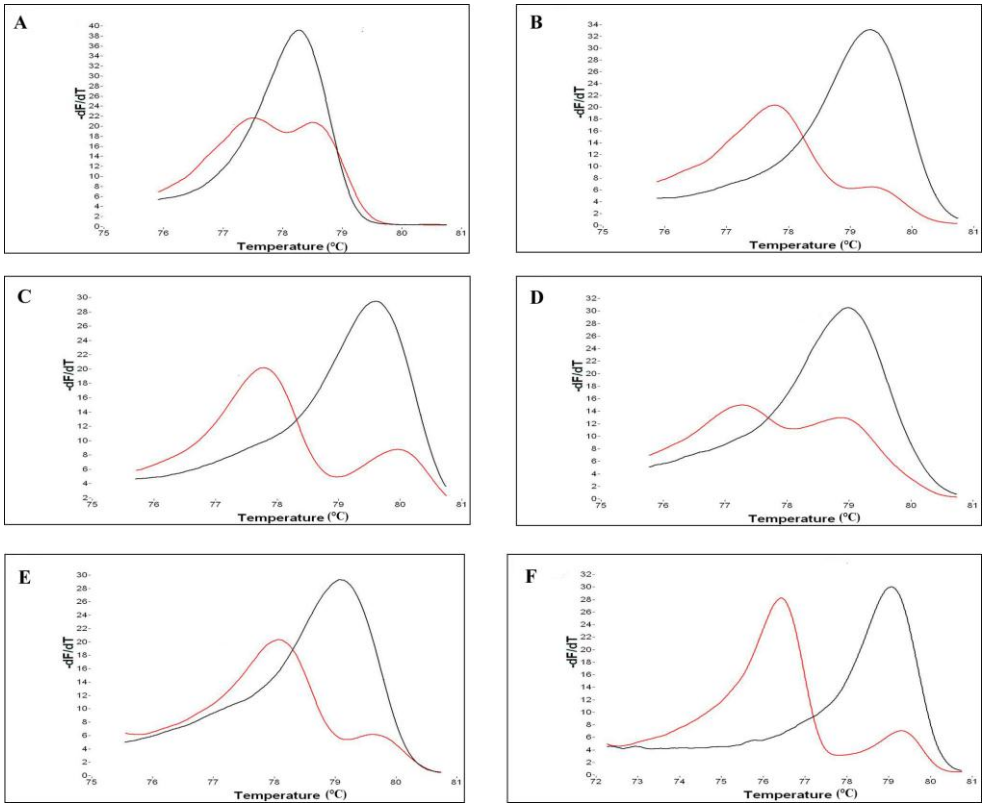

Figure 3-1 Avian gender ID HRMA of six species. Fluorescence derivative plots of melting curves are shown for – **A** Greater Roadrunner, **B** Lesser Flamingo, **C** Saddle-billed Stork, **D** Scarlet Ibis, **E** White-bellied Stork, and **F** Roseate Spoonbill. Melting curves of males (ZZ) are unimodal, whereas those of females (WZ) are bimodal. Each curve represents one individual.

All melting temperatures ranged from 72-81°C but curves are slightly variable among species due to inherent differences within sequences. For example, the profile of Greater Roadrunner has a melting temperature range of 76-80°C, which is low when compared to that of Roseate Spoonbill (72-81°C). Nevertheless, a general pattern can be observed. Females (WZ) can be easily distinguished by their bimodal heteroduplex curves from the unimodal homoduplex curves of males (ZZ). Unimodal melting curves

for male Marabou Stork are shown (**Figure 3-2**) but no DNA from females were available for comparison.

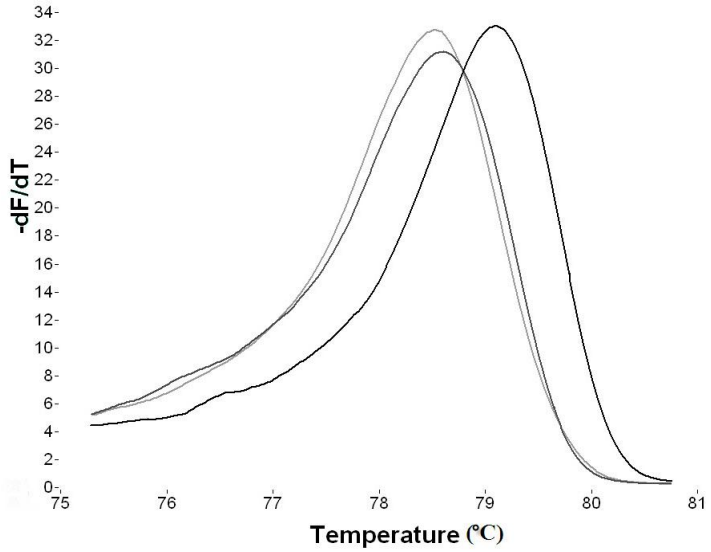

Figure 3-2 Avian gender ID HRMA of male Marabou Stork *(Leptoptilos crumenifer*). The fluorescence derivative plots of unimodal melting curves of males (ZZ) are shown. Melting curves of females (WZ) are bimodal (not shown). Each curve represents one individual.

Dr. Miguel A. Mora at Texas A&M University provided blood samples from Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*) (n = 12), for which specimens are listed in **Table 3-3**.

Table 3-3 Specimen list for blood samples from Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*). Samples were provided by Dr. Miguel A. Mora (Department of Wildlife and Fisheries Sciences (TAMU)). Highlighted in pink are specimens of whose molecular gender ID was in conflict with known gender.

	Specimen ID	Gender (Given)	Capillary # (HRM)	HRM Date (MM/DD/2011)	Gentoype (HRM)	Gender (HRM)	Multiplex PCR (Han *et al.* 2009)	Direct Sequencing	Remarks
1	SB3100	♂	19	03/24	Homoduplex	♂	♂	P2-P14	Confirmed ZZ
2	SB21168	♀	20	03/24	Heteroduplex	♀	♀	n/a	Confirmed WZ
3	SB3528	♀	21	03/24	Heteroduplex	♀	♀	n/a	Confirmed WZ
4	SB3178	♂	22	03/24	Homoduplex	♂	no amp	P2-P14	Confirmed ZZ
5	SB3321	♂	23	03/24	n/a	no amp	♂	P2-P14	Confirmed ZZ
6	SB3460	♀	24	03/24	Heteroduplex	♀	♀	P2-P0	Confirmed WZ
7	SB3543	♀	25	03/24	Heteroduplex	♀	♀	P2-P0	Confirmed WZ
8	SB3560	♀	26	03/24	Heteroduplex	♀	♀	n/a	Confirmed WZ
9	SB3179	♂	27	03/24	unk	unk	♂	P2-P14	Confirmed ZZ
10	SB2321	♂	28	03/24	Heteroduplex	♀	♂	n/a	Confirmed ZZ
11	SB3042	♂	29	03/24	Heteroduplex	♀	♂	n/a	Confirmed ZZ
12	SB3350	♀	30	03/24	Homoduplex	♂	♀	n/a	Confirmed WZ

A multiplex PCR assay that incorporated primers P8 F (Griffiths et al. 1998), P0 F (Han et al. 2009), and HRMSA-Avian-WZ-Rev was used to corroborate results obtained with HRMA. Electrophoresis through a 2.5% agarose gel was adequate to score gender based amplification partial CHD genes. Male Attwater's Prairie Chicken exhibited a band ≈279 bp in size when using conventional agarose gels and gender ID primers P8 and HRMSA-Avian-WZ-Rev, whereas females showed an additional fragment ≈114 bp longer (**Figure 3-3**), this corresponding to the amplicon of P0/P8 primer combination (see **Figure 1-2**).

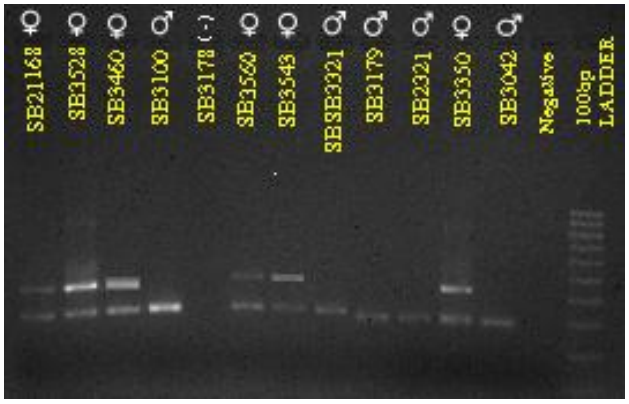

Figure 3-3 Amplification of Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*) using multiplex PCR. Fragments were visualized in a 2.5% Etbr-stained agarose gel. Multiplex PCR incorporated primers P0 F (Han et al. 2009), P8 F (Griffiths et al. 1998), and HRMSA-Avian-WZ-Rev. P2/P14 direct sequencing confirmed SB3178 as homozygous (male).

Use of the HRMSA-Avian-WZ-Rev primer instead of the more commonly used P2 R (Griffiths et al. 1998) for the multiplex assay allowed for gender ID to be confirmed based on a smaller fragment of the CHD alleles without sacrificing accuracy. Amplifying a shorter fragment would be advantageous if samples were degraded.

3.2 *Universal Application of HRMA for Avian Gender Determination*

After these preliminary trials with multiple avian species, primers HRMSA-Avian-WZ-Fwd and HRMSA-Avian-WZ-Rev were aligned against CHD-Z and CHD-W genes of 50 species in GenBank, representing a total of 15 families. Alignment revealed that the targeted region is highly conserved and thus of potential universal avian gender ID application. **Figure 3-4** shows the placement of the HRMSA-Avian-WZ forward and reverse primers along the 81-bp fragment of both W and Z chromosomes of 24

terrestrial avian species or ‘land birds’ and **Figure 3-5** shows the same for 26 aquatic species or ‘waterbirds’.

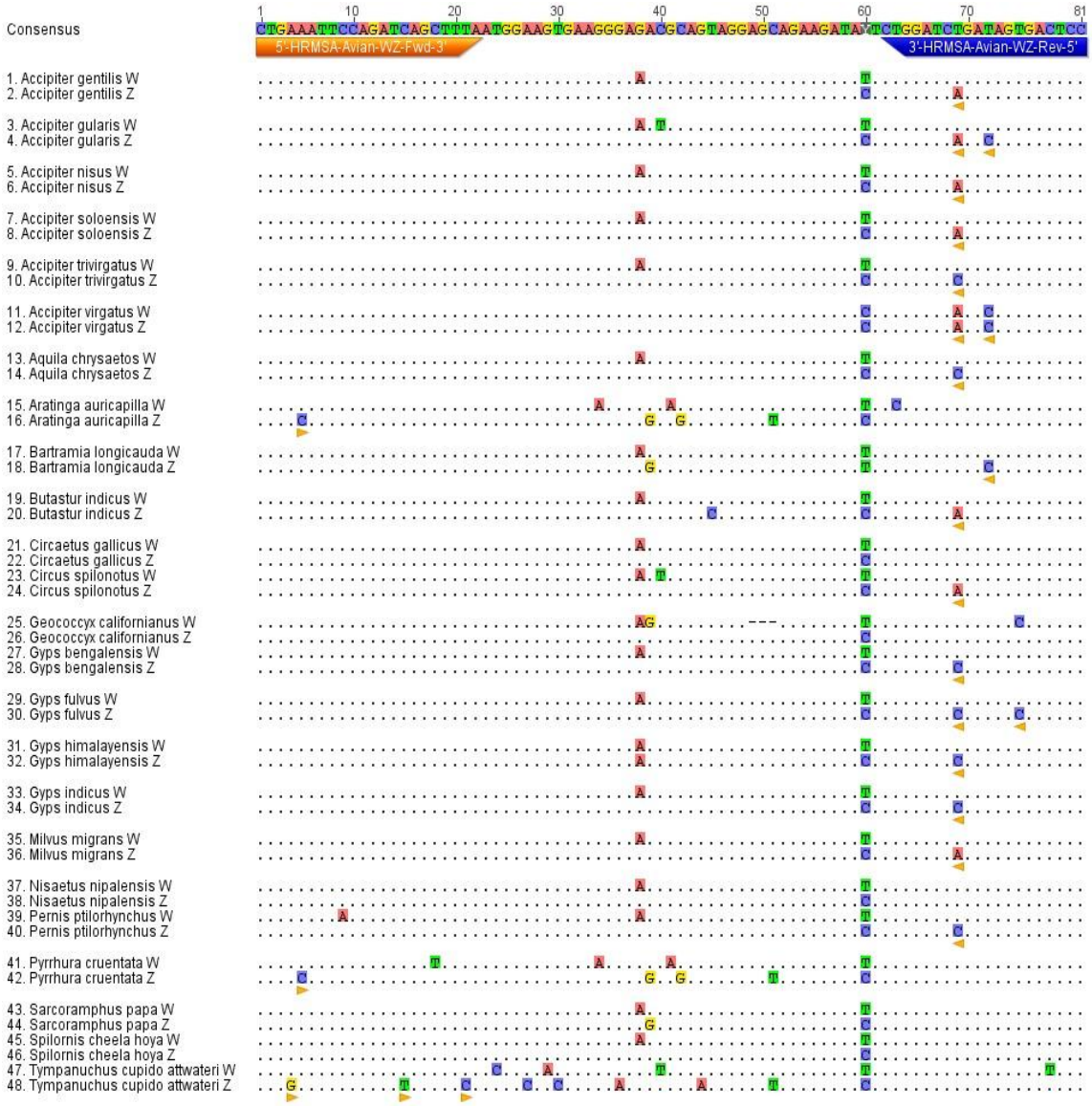

Figure 3-4 Sequences of CHD-Z and CHD-W amplified segments of various terrestrial avian species (n = 24). Differences (consensus) between the two alleles and the placement of the HRM short-amplicon (SA) Avian-WZ primer set are shown. Symbols (◄) under base pairs denote mismatches to primers in the CHD-Z indicating species that require specific primer(s).

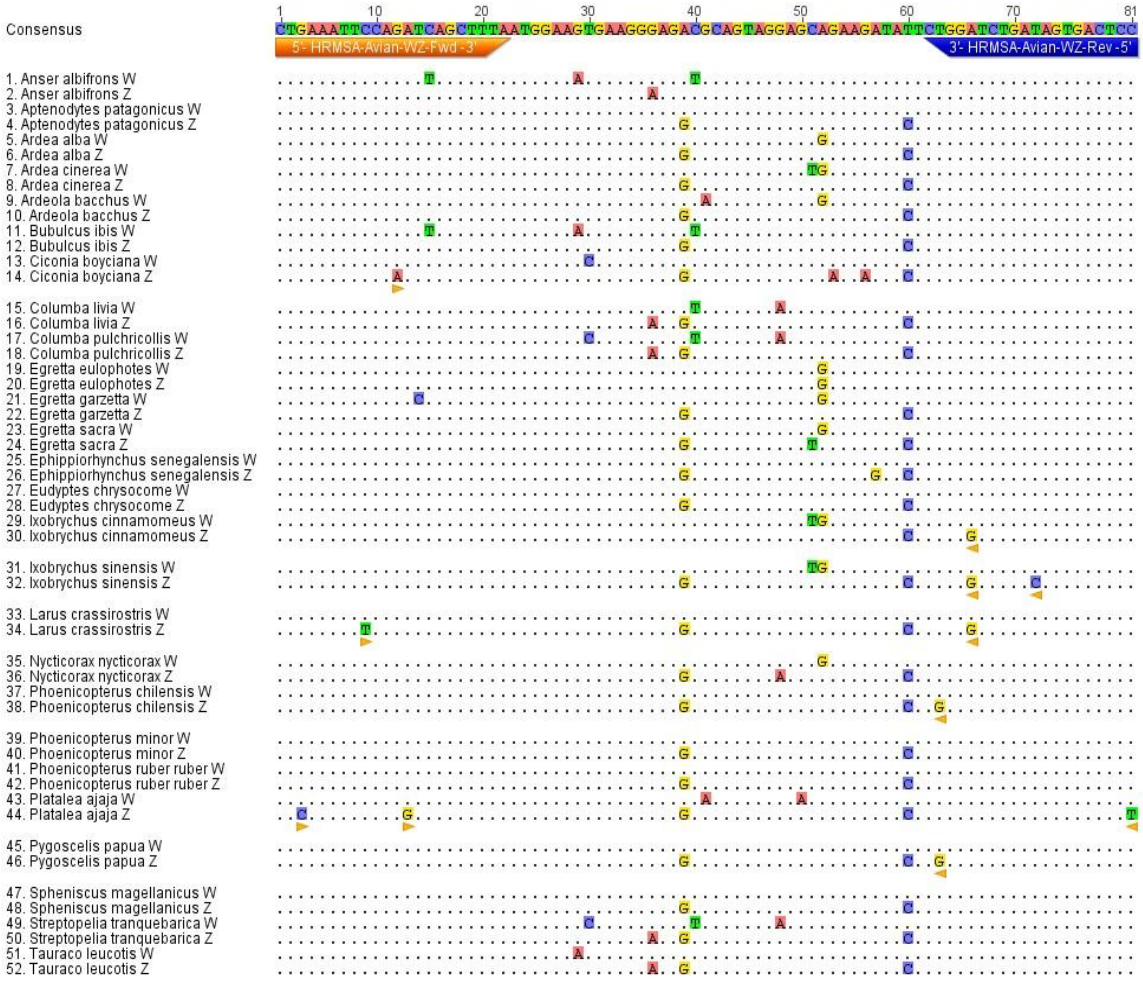

Figure 3-5 Sequences of CHD-Z and CHD-W amplified segments of various 'waterbird' species (n = 26). Differences (consensus) between the two alleles and the placement of the HRM short-amplicon (SA) Avian-WZ primer set are shown. Symbols (◄) under base pairs denote mismatches to primers in the CHD-Z indicating species that require specific primer(s).

Of the multiple sequence alignments of the CHD segment amplified with primers HRMSA-Avian-WZ forward and reverse, 19 species of terrestrial birds (**Figure 3-4**) and seven species of 'waterbirds' (**Figure 3-5**) have mismatches within the priming sites of the forward or the reverse primer. These instances are identified with symbols next to the CHD-Z of species that fall into this category, indicating that 'CHD-Z specific'

forward and/or reverse HRMSA primers are required for unambiguous HRMA gender ID assays. Accordingly, species, genus, or family-specific HRMSA primers are needed for gender ID (**Table 3-4**).

Table 3-4 Examples of taxon or species-specific HRMSA primer sets for gender ID. Their design was based on sequences of CHD-Z and CHD-W amplified segments of various avian species.

Family - Family name		Species	Common name	HRMSA forward primers (5' --> 3')	HRMSA reverse primers (5' --> 3')
Phasianidae - Pheasants, Grouse	1	*Tympanuchus cupido attwateri*	Attwater's Prairie Chicken	HRMSA-PrairieChicken-WZ-Fwd CTG GAA TTC CAG GTC TGC TTT A	HRMSA-PrairieChicken-WZ-Rev GGA ATC ACT ATC AGA TCC AG
Accipitridae - Hawks, Eagles, Vultures	2	*Accipiter gentilis*	Northern Goshawk	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	3	*Accipiter gularis*	Japanese Sparrowhawk	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	4	*Accipiter soloensis*	Chinese Sparrowhawk	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	5	*Accipiter trivirgatus*	Crested Goshawk	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	6	*Accipiter virgatus*	Besra Sparrowhawk	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	7	*Accipiter nisus*	Eurasian Sparrowhawk	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipiter nisus-WZ-Rev TTT CTT TCT GAG ACG GAG TC
	8	*Aquila chrysaetos*	Golden Eagle	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG TC
	9	*Butastur indicus*	Gray-faced Buzzard	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	10	*Circaetus gallicus*	Short-toed Snake Eagle	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	11	*Circus spilonotus*	Eastern Marsh-harrier	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	12	*Gyps bengalensis*	White-rumped Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	13	*Gyps fulvus*	Griffon Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	14	*Gyps himalayensis*	Himalayan Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Gyps himalay-WZ-Rev TTT CTT TTT GAG ATG GAG GC
	15	*Gyps indicus*	Indian Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	16	*Milvus migrans*	Black Kite	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	17	*Nisaetus nipalensis*	Mountain Hawk-Eagle	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	18	*Sarcoramphus papa*	King Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	19	*Pernis ptilorhynchus*	Oriental Honey-buzzard	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	20	*Spilornis cheela hoya*	Crested Serpent-eagle	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
Laridae - Gulls	21	*Larus crassirostris*	Black-tailed Gull	HRMSA-Larus crass-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Larus crass-WZ-Rev GGA GTC ACT ATC AGA GCC AG
Scolopacidae - Sandpipers	22	*Bartramia longicauda*	Upland Sandpiper	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Bart long-WZ-Rev GGA GTC ACT GTC AGA TCC AG
Spheniscidae - Penguins	23	*Pygoscelis papua*	Gentoo Penguin	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Pygo papua-WZ-Rev GGA GTC ACT ATC AGA TCC CG
Ciconiidae - Storks	24	*Ciconia boyciana*	Oriental Stork	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Avian-WZ-Rev GGA GTC ACT ATC AGA TCC AG
Threskiornithidae - Ibises, Spoonbills	25	*Platalea ajaja*	Roseate Spoonbill	HRMSA-Plata ajaja-WZ-Fwd CTC AAA TTC CAG ATC AGC TTT A	HRMSA-Avian-WZ-Rev GGA GTC ACT ATC AGA TCC AG
Ardeidae - Herons, Egrets, Bitterns	26	*Ixobrychus sinensis*	Yellow Bittern	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Ixo sinensis-WZ-Rev GGA GTC ACT GTC AGA CCC AG
	27	*Ixobrychus cinnamomeus*	Cinnamon Bittern	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Ixo cinna-WZ-Rev GGA GTC ACT ATC AGA CCC AG
Phoenicopteridae - Flamingos	28	*Phoenicopterus chilensis*	Chilean Flamingo	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Phoenicopterus-WZ-Rev TGA GAT GGA GTC ACT ATC AG
	29	*Phoenicopterus ruber ruber*	Caribbean Flamingo	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Phoenicopterus-WZ-Rev TGA GAT GGA GTC ACT ATC AG
	30	*Phoenicopterus minor*	Lesser Flamingo	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Phoenicopterus-WZ-Rev TGA GAT GGA GTC ACT ATC AG

	14 *Gyps himalayensis*	Himalayan Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Gyps himalay-WZ-Rev TTT CTT TTT GAG ATG GAG GC
	15 *Gyps indicus*	Indian Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	16 *Milvus migrans*	Black Kite	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	17 *Nisaetus nipalensis*	Mountain Hawk-Eagle	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	18 *Sarcoramphus papa*	King Vulture	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	19 *Pernis ptilorhynchus*	Oriental Honey-buzzard	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
	20 *Spilornis cheela hoya*	Crested Serpent-eagle	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Accipitridae-WZ-Rev TTT CTT TCT GAG ATG GAG GC
Laridae - Gulls	**21** *Larus crassirostris*	Black-tailed Gull	HRMSA-Larus crass-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Larus crass-WZ-Rev GGA GTC ACT ATC AGA GCC AG
Scolopacidae - Sandpipers	**22** *Bartramia longicauda*	Upland Sandpiper	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Bart long-WZ-Rev GGA GTC ACT GTC AGA TCC AG
Spheniscidae - Penguins	**23** *Pygoscelis papua*	Gentoo Penguin	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Pygo papua-WZ-Rev GGA GTC ACT ATC AGA TCC CG
Ciconiidae - Storks	**24** *Ciconia boyciana*	Oriental Stork	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Avian-WZ-Rev GGA GTC ACT ATC AGA TCC AG
Threskiornithidae - Ibises, Spoonbills	**25** *Platalea ajaja*	Roseate Spoonbill	HRMSA-Plata ajaja-WZ-Fwd CTC AAA TTC CAG ATC AGC TTT A	HRMSA-Avian-WZ-Rev GGA GTC ACT ATC AGA TCC AG
Ardeidae - Herons, Egrets, Bitterns	**26** *Ixobrychus sinensis*	Yellow Bittern	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Ixo sinensis-WZ-Rev GGA GTC ACT GTC AGA CCC AG
	27 *Ixobrychus cinnamomeus*	Cinnamon Bittern	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Ixo cinna-WZ-Rev GGA GTC ACT ATC AGA CCC AG
Phoenicopteridae - Flamingos	**28** *Phoenicopterus chilensis*	Chilean Flamingo	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Phoenicopterus-WZ-Rev TGA GAT GGA GTC ACT ATC AG
	29 *Phoenicopterus ruber ruber*	Caribbean Flamingo	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Phoenicopterus-WZ-Rev TGA GAT GGA GTC ACT ATC AG
	30 *Phoenicopterus minor*	Lesser Flamingo	HRMSA-Avian-WZ-Fwd CTG AAA TTC CAG ATC AGC TTT A	HRMSA-Phoenicopterus-WZ-Rev TGA GAT GGA GTC ACT ATC AG

Since gender ID using HRMA based on SNPs relies on discerning heteroduplex curves of females from homoduplex curves of males, mismatches within the primers along the CHD-Z portion of the gene would generate heteroduplex melting curves in males, rendering the assay ambiguous. HRMA relies on perfect match within primers to distinguish homozygotic males from females. However mismatches in the CHD-W region of the primers, as long as these do not prevent amplification, will not affect gender ID, provided that flanked sequences of W and Z fragments differ from each other.

Primers HRMSA-Avian-WZ-Rev and HRMSA-Avian-WZ-Fwd were not successful in diagnosing gender since melting curves for male Attwater's Prairie Chicken were not distinguishable from melting curves of females (**Figure 3-6**).

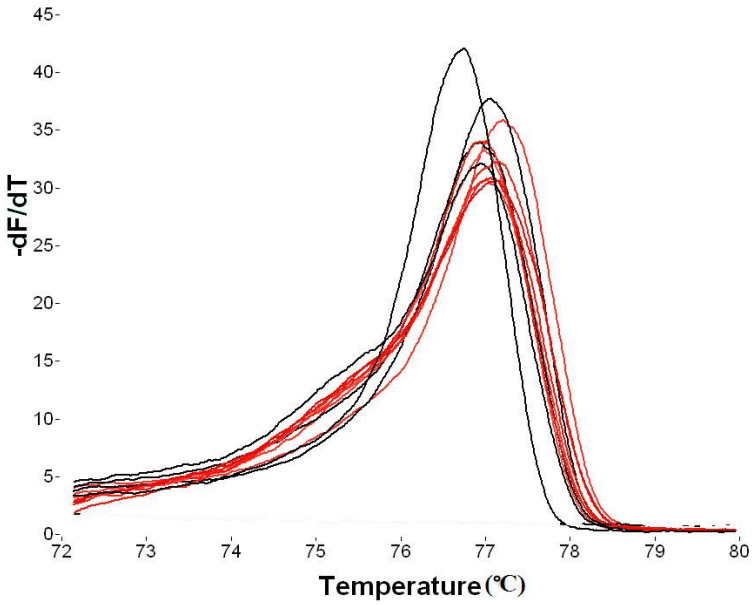

Figure 3-6 Avian gender ID HRMA of Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*) with original short-amplicon primers. Fluorescence derivative plots of males (black) and females (red) are shown. Melting curves are not diagnostic of gender. Each curve represents one individual.

The alignment of the targeted segment (**Figure 3-4**) reveals three SNPs in the HRMSA-Avian-WZ-Fwd primer for the CHD-Z gene so two new primers, HRMSA-Prairie Chicken-WZ-Fwd and HRMSA-Prairie Chicken-Rev (**Table 3-4**) were designed based on the CHD-Z and CHD-W sequences of this species. These sequences were generated from PCR-amplified products using primers P2/P14 for males and P0/P2 for females.

The melting protocol entailed a ramp rate of 0.3°C/sec, data acquisition beginning at 65°C, a final temperature of 80°C, and cooling to 59°C. Derivative plots of fluorescence against melting temperatures were generated using HR-1 Melting Analysis

tools and revealed an atypical melting curve pattern (**Figure 3-7**). Note that it is important to incorporate positive controls to confirm the results of HRMSA.

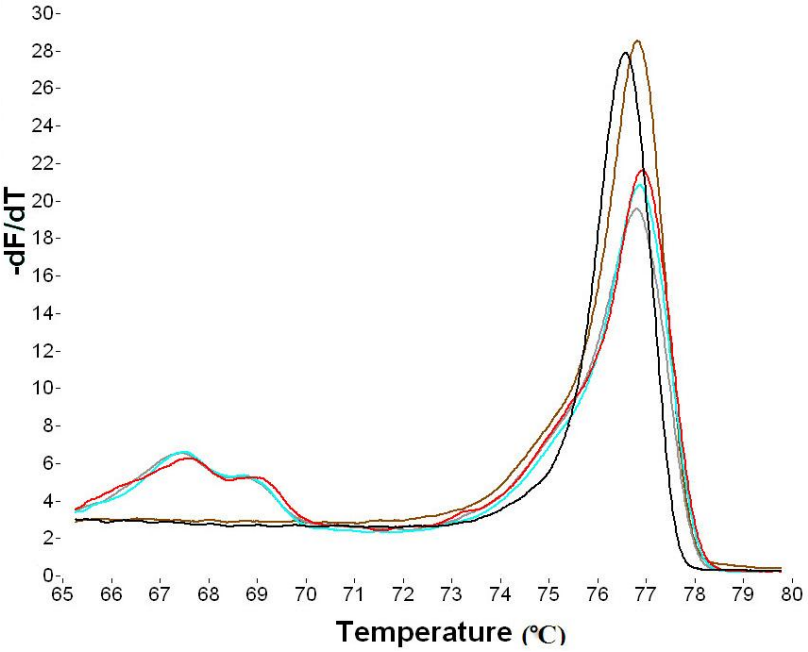

Figure 3-7 Avian gender ID HRMA using species-specific primers for Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*). Fluorescence derivative plots of unimodal melting curves of males (ZZ) and multiple melting domains of females (WZ) are shown. Each curve represents one individual.

Similarly, it was not possible to distinguish between male and female Chilean Flamingo using primers HRMSA-Avian-WZ-Fwd and HRMSA-Avian-WZ-Rev (**Figure 3-8**).

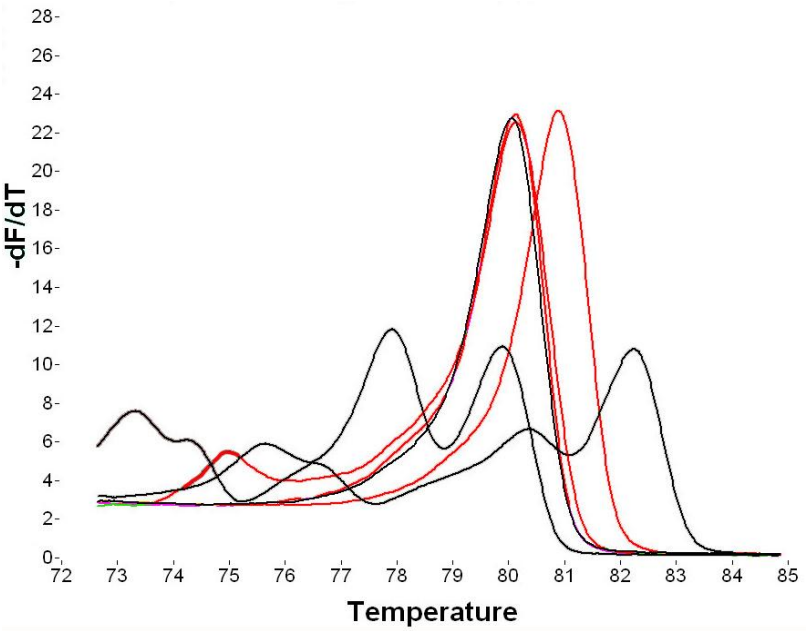

Figure 3-8 Avian gender ID HRMA of Chilean Flamingo (*P. chilensis*) with original short-amplicon primers. Fluorescence derivative plots of melting curves of males (black) and females (red) are shown. Melting curves are not diagnostic of gender. Each curve represents one individual.

Sequencing of CHD-W and CHD-Z revealed a mismatch (G at the second position from the 3' end) in the HRMSA-Avian-WZ-R primer for the CHD-Z gene (**Figure 3-5**). However, a single SNP in the HRMSA-Avian-WZ-R primer at the second position from the 3' end along the CHD-Z gene appears to prevent the amplifications of the CHD-Z allele (**Figure 3-5**). Mutations near the 3' end of a primer are capable of preventing primer extension (Rychlik 1995)

A species-specific HRMSA reverse primer (5'-GGA GTC ACT ATC AGA TCC CG-3') for Chilean Flamingo was synthesized to be used together with HRMSA-Avian-WZ-Fwd. The results of this HRMA still yielded no marked pattern differentiating gender (**Figure 3-9**).

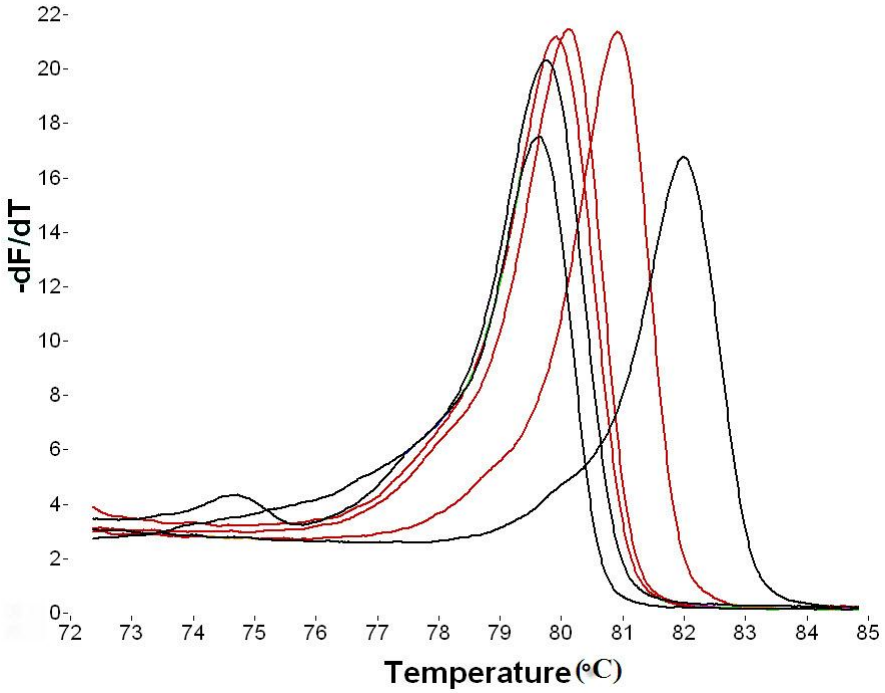

Figure 3-9 Avian gender ID HRMA for Chilean Flamingo (*P. chilensis*) with species-specific primers. Fluorescence derivative plots of melting curves of males (black) and females (red) amplified using species-specific short-amplicon primers are shown. Melting curves are not diagnostic of gender. Each curve represents one individual.

In an attempt to correct the misidentification of Chilean Flamingo as well as to produce a diagnostic assay that could be used for all Flamingos, a new HRMSA-*Phoenicopterus*-WZ-Rev primer (5'-TGA GAT GGA GTC ACT ATC AG-3') was designed (**Table 3-4**) located 6-bp upstream from the original (**Figure 3-10**).

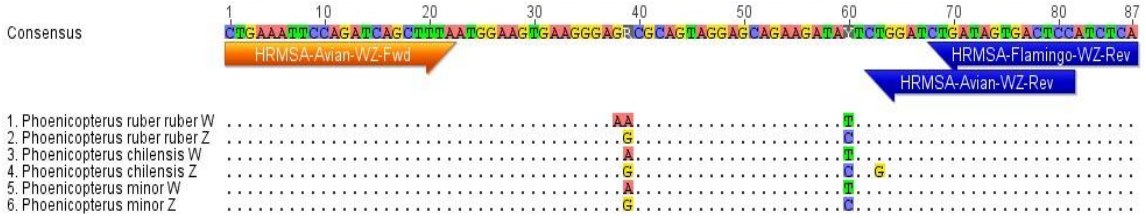

Figure 3-10 Sequence of CHD-Z and CHD-W amplified segments of Caribbean Flamingo (*P. ruber ruber*), Chilean Flamingo (*P. chilensis*), and Lesser Flamingo (*P. minor*). Differences (consensus) between the two alleles and the placement of the HRMSA primer set and HRMSA-Flamingo-WZ-Rev primer are shown.

The gender of all three *Phoenicopterus* species (*P. minor*, *P. ruber ruber*, and *P. chilensis*) assayed was correctly diagnosed. Heteroduplex melting curves for females are shown in **Figure 3-11A** and homoduplex melting curves for males in **Figure 3-11B**. The new Flamingo-specific primer set effectively eliminated the need for multiple primer sets for these three species.

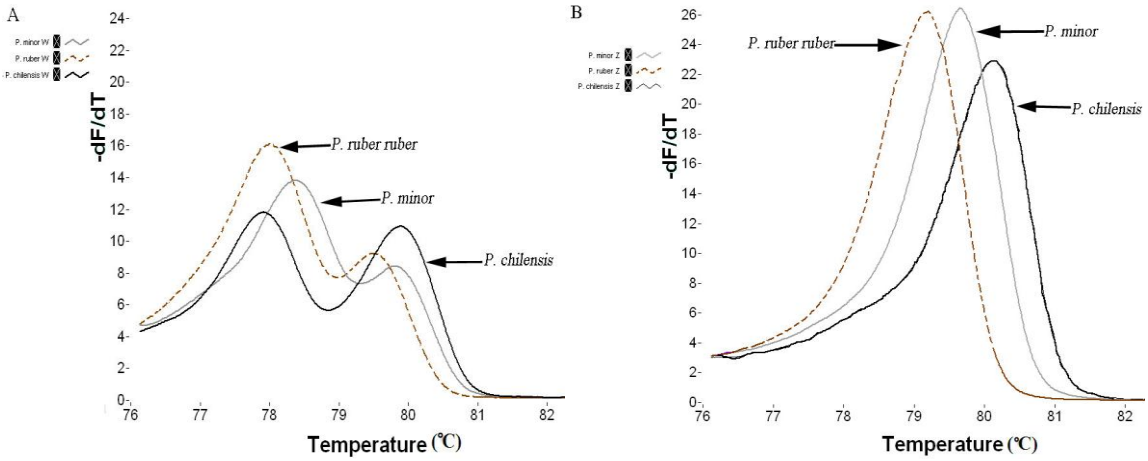

Figure 3-11 Avian gender ID HRMA for three Flamingo species with genus-specific primers. Fluorescence derivative plots of melting curves are shown for **A** Female Lesser Flamingo (*P. minor*), Caribbean Flamingo (*P. ruber*), and Chilean Flamingo (*P. chilensis*) and **B** Male Lesser Flamingo (*P. minor*), Caribbean Flamingo (*P. ruber*), and Chilean Flamingo (*P. chilensis*). Melting curves of males (ZZ) are unimodal, whereas those of females (WZ) are bimodal. Each curve represents one individual.

3.3 *Discussion of Universal Molecular Gender ID of Birds*

While Attwater's Prairie Chicken required species-specific primers, diagnostic melting curves for Greater Roadrunner (*Geococcyx californianus)* (**Figure 3-1A**) were obtained despite the presence of one mismatch at the seventh position from the 5' end of the reverse primer (**Figure 3-4**). Santamaria et al. (2010) used several novel primer combinations to ID gender of Greater Roadrunner and reported one instance of preferential amplification along both the CHD-W gene of CHD-Z, misidentifying one female control specimen as a male. They also observed preferential amplification of the CHD-W allele but it was not an issue since presence of the amplicon corresponding to CHD-W always identifies females. Because HRMA relies on SNPs to distinguish homozygotic males from females, primers corresponding to the CHD-Z allele must be a perfect match. Mismatches in either the HRMSA-Avian-WZ forward or reverse primer in the CHD-W allele however, will not necessarily affect gender ID. Consequently, scoring may be misleading in instances where mismatched nucleotides of primers cause a null allele for CHD-W, resulting in misclassification of heterozygotic females as homozygotic males (Carlson et al. 2006).

Where gender ID for most species was diagnosed by bimodal melting curves for females (WZ), curves for female Attwater's Prairie Chicken had multiple melting domains. In a derivative plot of temperature and fluorescence, melting curves of females included weaker bimodal peaks between 65°C and 70°C as well as a stronger unimodal peak near 76°C. Melting curves of male Attwater's Prairie Chicken were unimodal producing only a homoduplex around 76°C (**Figure 3-7**).

Primers with mismatches close to the 3' end are likely causes when amplifications fail, especially when two or more are located within the last 4 bp, whereas mutations at a primer's 5' end are less likely to inhibit the reaction (Rychlik 1995). Gender ID of Roseate Spoonbill was successful using HRMSA-Avian-WZ forward and reverse primers (**Figure 3-1F**) despite mismatched primers for the CHD-Z allele. Two mutations occur in the HRMSA-Avian-WZ-Fwd primer (at the third and thirteenth nucleotides from the 5' end) and one mutation occurs in the HRMSA-Avian-WZ-Rev primer (at the first nucleotide position from the 5' end) (**Figure 3-5**). This issue will be further discussed in the subsequent section, specifically how to proceed when an assay requires alternate primers.

The presence of multiple melting domains in curves of male Chilean Flamingo and homoduplex curves in females (**Figure 3-8**) could not be explained since amplicons with species-specific primers failed to produce distinctly diagnostic melting curves. Caution must be taken when gender ID of large samples is carried on without validating the assay first (Dawson et al. 2001). For instance, amplification strength of CHD-W and CHD-Z may vary between individuals because of DNA quality (Fridolfsson, A.K. and H. Ellegren 1999) and can result in genotyping errors.

It is important to note that gender ID of species in the Family Accipitridae is particularly problematic since the intron length differences between the CHD-W and CHD-Z alleles are only ≈3-9 bp long. In addition, sequences for the CHD gene are not as conserved as other birds (Chou et al. 2010). Using CHD-W specific and common TaqMan probes (Chou et al. 2010) in combination with P2 R and P8 F (Griffiths et al.

1998) successfully determined gender of eight species and concluded that the probes would be useful for seven other Accipitrid species indicated by sequence alignments and placement of the probes therein. Gender ID using the HRMSA-Avian-WZ-Fwd primer for the 19 species within the Family Accipitridae listed in **Figure 3-4** is possible if the reverse primer is moved 14 nucleotides upstream from HRMSA-Avian-WZ-Rev. This region is considerably more conserved than the targeted TaqMan probes and CHD-W specific sites. With the exception of *Gyps himalayensis* and *Accipiter nisus*, a HRMSA-Accipitridae-WZ-Rev primer (**Table 3-4**) could identify gender of multiple species of raptor except for Besra Sparrowhawk (*A. virgatus*). The 95-bp fragment for *A. virgatus* is identical for both W and Z alleles, suggesting a submission error to GenBank. However, if the sequences for CHD-W and CHD-Z alleles of *A. virgatus* are correct, the assay using the Accipiteridae-specific primer would not be diagnostic, as both would be scored as males. Even though adult female Besra Sparrowhawk are markedly larger in size than males (Huang et al. 2004), monitoring sex ratio and gender ID of chicks is still important in order to evaluate their population stability (Chou et al. 2010).

3.4 *General Recommendations of Applying HRMA to ID Gender of Birds*

In this study we designed a series of primer sets that can be assayed to validate gender from avian samples. If avian CHD binding-gene sequences are not available for both female and male individuals of the target species, they can first be readily obtained by PCR amplification with P14 F, P0 F, and P2 R. Gender-validated samples are desirable for this purpose, but multiple alignments against existing sequences in

GenBank provide a means to evaluate the fit of the HRMSA-Avian-WZ-Fwd and HRMSA-Avian-WZ-Rev primers. It is important to keep in mind that, for accurate gender ID, both HRM primers must be a perfect match to the CHD-Z gene. **Figure 3-12** depicts a flow chart of how to proceed to select HRMSA primers. **Table 3-4** lists supplementary examples of primer sets tailored for the group or individual species.

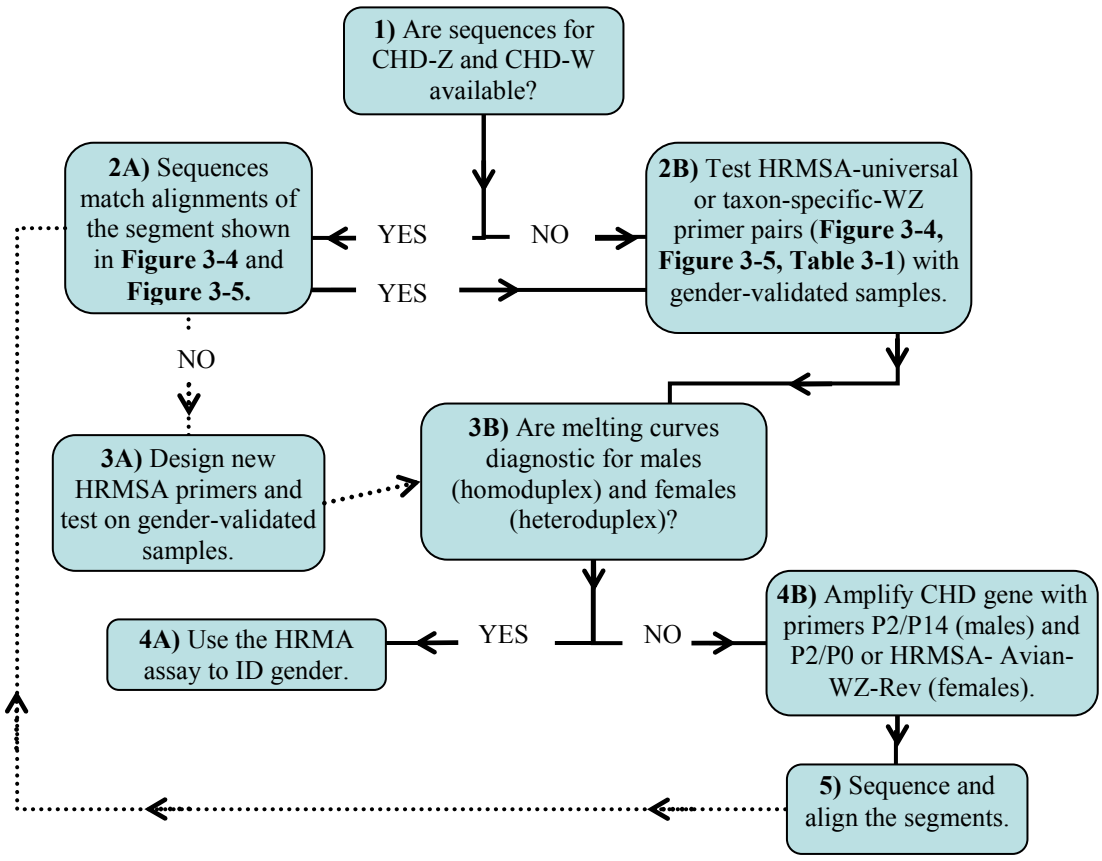

Figure 3-12 Flowchart depicting the basic protocol and process of trouble-shooting and optimizing molecular assays for avian gender ID based on HRM. Arrows indicate the direction of progress to subsequent steps.

Custom-made HRMSA primers for avian gender ID have the benefit of drastically reducing time, cost, and error rate of scoring, all which are relevant for researchers and zoologists likely working with natural populations of threatened or endangered avian groups e.g. members of the Family Accipitridae or the Genus *Phoenicopterus*. The HRMA assays developed here are fast and economical. From start to finish, a sample of blood or feather-bulb tissue to scoring analysis, positive gender ID can be completed in less than two hours for less than 40 cents per sample.

4. CONSERVATION OF A WILD POPULATION OF NESTING CARIBBEAN FLAMINGO IN YUCATÁN, MEXICO

4.1 *Protection and Management*

As part of a mission to promote conservation projects, a non-governmental organization (NGO) called Niños y Crías A.C. in Yucatán, Mexico has been designing programs intended primarily for environmental education for younger generations. Children can learn about local wildlife, responsible waste management, fire prevention, and vegetation and habitat renewal (Niños y Crías A.C. 2012). This NGO believes that the best way to spread awareness in sustainable practices is through local participation of communities in the preservation of the Ría Lagartos and Ría Celestun Biosphere Reserves along the Yucatán Gulf Coast. Beginning in 1999, a program aimed to protect sites for nesting, food, and freshwater supplies of Caribbean Flamingo, has given residents the opportunity to experience conservation in action.

Every year Niños y Crías A.C. organizes a banding event that draws local volunteers of all ages, international and national program directors, wildlife researchers, animal husbandry experts, veterinary staff, and media to aide in the project. Participants are involved in some part of the process: corralling, weighing and measuring, obtaining crop and blood samples, diagnosing and treating health issues, and gently returning each Caribbean Flamingo fledgling to the lagoon.

Niños y Crías A.C. also collaborates with Mexican Federal and State authorities to monitor survivorship, birth rates, migratory patterns, and bands roughly 8% of the total population of nestling juveniles throughout the year. The plastic bands inscribed with a

unique 4-letter code are attached to the right leg of fledgling Caribbean Flamingo (**Figure 4-1**) and can be easily read through a telescope for census by boat.

Figure 4-1 Plastic band placed on juvenile Caribbean Flamingo (*P. ruber ruber*). Each is inscribed with a unique 4-letter code and is attached to the right leg.

Active habitat restoration, like that of the Punta Mecoh zone damaged by Hurricane Wilma, has successfully drawn thousands of Caribbean Flamingo back to nest safely ever since. This location is one of only four main breeding sites of this species and the most important site for the Yucatán population. This underscores the need for conservation of the surrounding marshes and lagoons.

4.2 *Field Sampling of DNA and Molecular Methods*

Samples collected from individual fledgling Caribbean Flamingo during annual banding events numbered 429 feather and 42 blood (August of 2009), 338 feather (2010), and 177 feather and 26 blood samples (September 2011). Only the cohort sampled in 2009 has been analyzed since importers are required to carry them.

The breeding colony of Caribbean Flamingo is monitored and the development of chicks is recorded. This information is used to determine the date of the banding event is set and preparation begins. The event begins before sunrise when the nesting colony has not yet dispersed to feed. This maximizes the chance of banding as many individuals as possible, the ordeal must take place in the dark of early morning. Two or three corrals with weight and measurement stations on either side, a veterinary post, and a pen for birds to rest (in case they become over-stressed) are constructed the day before on the just on the outskirts of the colony (**Figure 4-2**).

Figure 4-2 Corrals for fledgling Caribbean Flamingo (*P. ruber ruber*) along the shoreline of the Ría Lagartos Biosphere Reserve, Yucatán, Mexico.

Volunteers carry each individual for measurements of body size like mass, length of tarsus, head-and-bill, and flattened wing length, which recorded along with the code on the metal rings and plastic bands they receive (**Figure 4-3**).

Figure 4-3 Handling of juvenile Caribbean Flamingo (*P. ruber ruber*). Volunteers record measurements of tarsus length (left) and head-and-bill length (right).

If the fledgling appeared to be healthy and coping well with the handling procedures, both blood and non-primary feathers were sampled. Blood was not drawn from the very small-bodied fledglings. Instead, a few feathers were plucked to obtain tissue from the calamus for molecular gender ID. The last step before being released is application of the metal ring and plastic band. Each bird receives a plastic band with a unique 4-letter code on the right leg and a smaller metal ring specifying the Niños y Crías A.C. contact information on the left leg (**Figure 4-4**).

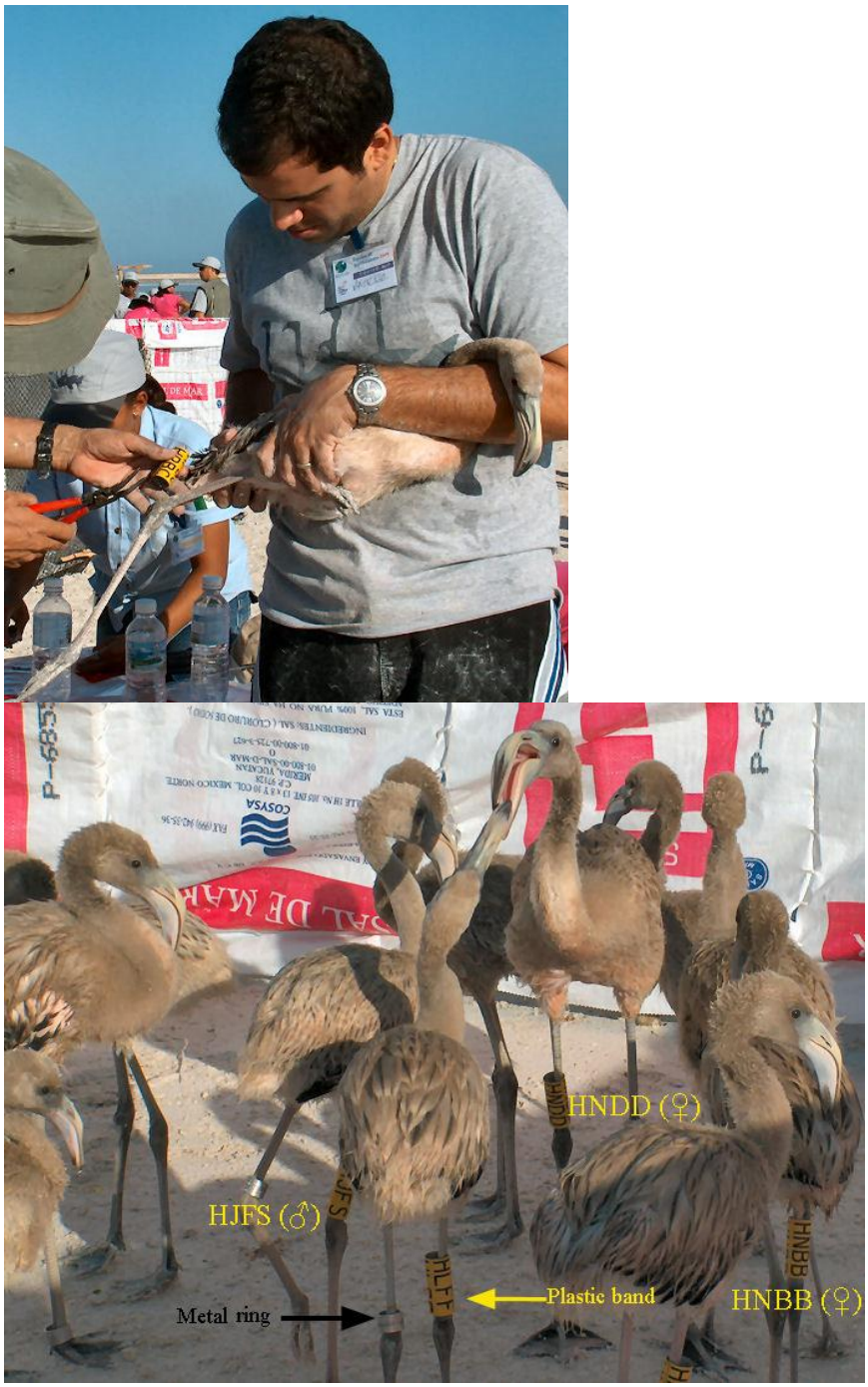

Figure 4-4 Banding of juvenile Caribbean Flamingo (*P. ruber ruber*). A plastic band with a 4-letter code is placed on the right leg and a metal ring with program contact information is placed on the left leg just before the juvenile is released to return to the nesting site (top). Gender identified in this study is shown in yellow as an example of characterization after feather sample analysis (bottom).

4.2.1 *DNA Extraction and PCR Amplification*

DNA extractions were carried out as described in Section 2.2.1. PCR amplifications and melting analyses were carried in a Light Cycler® 480 RT PCR System (Roche). Because the HRMA gender ID assay is highly accurate and amendable to high throughput, the Light Cycler® 480 could be used to process large sample sizes.

Reactions were prepared in 10 µl of final volume in 96-well TempPlates® (USA Scientific, Inc.) containing 2.5 µl ddH_2O, 0.5 µl LCGreen®Plus, 0.5 µl of each primer (HRMSA-Avian-WZ-Fwd and HRMSA-Avian-WZ-Rev), 5 µl 2x EconoTaq® PLUS polymerase (Lucigen), and 1 µl of isolated DNA template.

An initial denaturing step of 10 minutes at 95°C was followed by 70 cycles of strand denaturation at 94°C for 10 seconds, primer annealing at 50°C for 30 seconds, and extension at 72°C for 10 seconds. Negative controls (no DNA) were run with every reaction and standard precautions were taken to prevent contamination.

4.3 *Results*

Derivative plots of fluorescence against melting temperature (not shown) were generated for 326 individuals collected from the 2009 banding event in the Ría Lagartos Biosphere Reserve. The gender of 33 (9%) of the entire sample (n = 359) could not be determined because of low DNA content or quality resulting in failed amplifications (for detailed specimen list, see **Appendix Table A-1**). A total of 175 females and 151 males, which corresponds to a female to male ratio of 1.15, were identified. This proportion however, is not significantly different from unity ($\chi^2 = 1.767$) expected from a healthy

population. Multiple cohorts across seasons and accurate measurement of pertinent environmental parameters that could affect gender allocation are needed to determine whether sex manipulation exists in Caribbean Flamingo as suggested by Bertault et al. (2000) for Greater Flamingo.

4.4 *Applications of Gender Identification in Birds*

Few studies that monitor sex ratio in offspring of wild bird populations exist. In organisms whose gender is chromosomally determined, the sex ratio is not expected to deviate from unity. However, in some bird populations, this ratio has been reported to differ from expected. This is reputedly due to environmental pressures like food availability, sparse nesting habitat, or lack of rainfall, suggesting they are mechanisms of sex ratio manipulation (Bertault et al. 2000).

The Greater Flamingo (*Phoenicopterus ruber roseus*), a close relative of Caribbean Flamingo, appears to be able to manipulate sex ratios in response to fluctuating environmental conditions during the breeding season. Bertault et al. (2000) found a significant indication that more male Greater Flamingo nesting in Camargue, France, a colony that has been studied for more than thirty years, are produced early in the season. The relationship between size and gender can be studied by matching the weight of 3-5 month-old chicks at the time of banding and molecular gender ID by blood samples 6-8 months later. Males were found to be larger than females and according to the authors of that study, a strong positive correlation exists between older nestlings and

the likelihood to be male, a relationship that suggests maternal sex manipulation (Bertault et al. 2000).

While the basis of this biological mechanism is not well understood, Bertault et al. (2000) suggested that under favorable conditions, generally at the beginning of the nesting season, parents produce more males than females. Evolutionary hypotheses have been advanced to account for this bias in terms of the competitive advantage of the larger males over females and may be a result of faster-developing male-producing follicles and higher selective pressures, like mate competition, on males (Bertault et al., 2000).

Nager et al. (1999) experimentally manipulated conditions of female wild Lesser Black-backed gulls (*Larus fuscus*) by egg removal (to induce continuous egg-laying) and by providing food supplements. Egg production is costly and since males of this species are larger and grow faster, they require more energy and parental investment. Under meager conditions and continuous egg loss, male chick survival of unsupplemented females decreased dramatically. Survival ratio of both genders remained constant in supplemented females, despite egg removal.

Characterizing gender of sexually dimorphic adult birds is much simpler than establishing sex ratio of juveniles or sexually monomorphic birds and has been widely impractical on a large-scale (Komdeur 2004). Some levels of sex ratio bias are assumed to be a product of differential costs between producing males and females but this is not applicable to monogamous, sexually monomorphic, cooperatively breeding species. The

evolutionary theory behind local resource enhancement is assumed to impact sex ratio of local populations with these attributes (Ewen et al. 2001).

Female and male Red-cockaded Woodpeckers, and many other avian species, share parental care duties and adults and juveniles return to nest in the same colony each year. Also comparable to Flamingos, adult Red-cockaded Woodpeckers are cooperative breeders. 'Helpers', often inexperienced relatives or individuals that have lost their own offspring, commonly assist breeding pairs. One concept explored is that more parental investment in one sex is an advantage when that same sex contributes to future survival. For Red-cockaded Woodpecker in South Carolina, the population was male-biased and males are almost always the one to take on the 'nanny' role (Gowaty and Lennartz 1985).

Having accurate information about the demographics of both wild and captive populations is relevant to improve conservation practices of birds as well. It is especially important to manage sex ratio of threatened or endangered species since populations can be small. An unfavorable ratio of males to females along with very few contributing members to the breeding population has the potential to diminish the effective population size beyond recovery. If gender-biased mortality is observed, appropriate action can be taken by managers. For critically endangered species, maintaining an appropriate ratio of males to females is the key to successful breeding programs and conservation concerns. For example, little is understood about the ecology of the Black-eared Miner (*Manorina melanotis*), a sexually monomorphic bird species whose habitat in southern Australia is under threat of fragmentation by land-clearing and agricultural

activity. It is known that non-breeding male helpers are primary caregivers and females make few trips to feed nestlings. In species that produce an excess of one gender, it is usually biased towards production of the dispersing gender in order to avoid competition for scarce resources. Understanding the correlation between sex ratio manipulation and resource and spatial limitation in endangered birds represents a valuable opportunity for managers who may actively manipulate gender ratios or habitat parameters (Ewen et al. 2001).

Protected Natural Areas (PNAs) like the Biosphere Reserves in Yucatán are arguably some of the best preserved aquatic sites for the conservation and protection of wild animal species (Niños y Crías A.C. 2012) and offer opportunities to research elusive aspects of behavioral ecology and population dynamics. These studies are even more realistic with the recent advent of molecular techniques. The high-throughput molecular gender ID method developed here allows accurate quantification of the male to female ratio of juvenile Caribbean Flamingo, making it possible to correlate the seasonal success of the breeding population with the quality of their protected habitat and the seasonal stability of the population. The most informative and accurate statistics about the dynamics and reproductive success of any avian population, especially species that are migratory, is obtained from sampling juveniles at natal locations. Because fledglings are unable to fly, corralling and handling is less difficult than capturing adults, whose natal origins are unknown.

Of particular significance is the fact that observers who sight banded Caribbean Flamingo whose gender was determined are able to confirm their natal origin and age.

This data pooled with body measurements and gender ID from successive years of banding records will be used to study other sex-linked life history characters such as age at first breeding, longevity, migration pathways, and gender mediated dispersal. This is a study that has not yet been done for Caribbean Flamingo and is equally valid to other species vulnerable to population declines.

5. CONCLUSIONS

5.1 *Summary*

Methods to identify gender are essential management tools that provide insight into the population dynamics and structure of wild and captive birds. We have designed a novel, fast, and highly sensitive closed-tube HRMA method to identify the gender of Caribbean Flamingo and other birds from the characteristic shapes of homozygous (homoduplex) and heterozygous (heteroduplex) PCR-products. This method relies on short SNPs along a short 81 bp segment of the CHD gene and thus differs from other molecular assays to date that rely on size polymorphisms associated with introns to score gender. We demonstrate the utility of this approach on Caribbean Flamingo (*Phoenicopterus ruber ruber*), Lesser Flamingo (*P. minor*), Saddle-billed Stork (*Ephippiorhynchus senegalensis*), Scarlet Ibis (*Eudocimus ruber*), White-bellied Stork (*Ciconia abdimii*), Roseate Spoonbill (*Platalea ajaja*), Marabou Stork (*Leptoptilos crumeniferus*), and Greater Roadrunner (*Geococcyx californianus*). Moreover, specific assays were designed for other species that could not be diagnosed with the avian universal primers, namely Prairie Chicken (*Tympanuchus cupido attwateri*) and Chilean Flamingo (*P. chilensis*). Additional primer sets were designed for 15 avian families and guidelines for using HRMA for gender ID are detailed. This gender ID method has potential 'universal' application for non-ratite birds using a sample of blood or feather-bulb tissue and can be completed in less than two hours.

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APPENDIX A

Table A-1 Specimen list for feather and blood samples from fledgling Caribbean Flamingo (*Phoenicopterus ruber ruber*) collected during Niños y Crías A.C. banding in 2009. Specimen ID refers to the individual's unique ring-code.

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
1	HHAA	3126	Blood, Feather	Feather	62	06/02/10	Heteroduplex	♀	1.3g
2	HHAB	3127	Blood, Feather	Feather	85	06/03/10	Homoduplex	♂	1.5g
3	HHAC	3128	Blood, Feather	Feather	58	06/02/10	Homoduplex	♂	1.6g
4	HHAD	3129	Feather	Feather	74	02/11/11	Homoduplex	♂	
5	HHAF	3130	Feather	Feather	75	02/11/11	Homoduplex	♂	
6	HHAH	3131	Feather	Feather	76	02/11/11	Homoduplex	♂	
7	HHAZ	3140	Feather	Feather	77	02/11/11	Heteroduplex	♀	
8	HHBA	3141	Blood, Feather	Blood	78	02/11/11	Homoduplex	♂	
9	HHBB	3142	Feather	Feather	79	02/11/11	Heteroduplex	♀	
10	HHBC	3143	Feather	Feather	80	02/11/11	Homoduplex	♂	
11	HHBF	3145	Feather	Feather	n/a	04/12/11	Homoduplex	♂	
12	HHBH	3146	Feather	Feather	82	02/11/11	Homoduplex	♂	
13	HHBJ	3147	Blood, Feather	Blood	83	02/11/11	Homoduplex	♂	
14	HHBL	3148	Feather	Feather	84	02/11/11	Heteroduplex	♀	
15	HHBN	3149	Blood, Feather	Blood	85	02/11/11	Homoduplex	♂	
16	HHBO	3144	Feather	Feather	n/a	04/12/11	Homoduplex	♂	
17	HHBP	3150	Feather	Feather	6	06/03/10	Homoduplex	♂	
18	HHBS	3151	Blood, Feather	Feather	63	06/02/10	Heteroduplex	♀	
19	HHBT	3152	Feather	Feather	22	06/15/10	Homoduplex	♂	
20	HHBZ	3155	Feather	Feather	31	06/15/10	Homoduplex	♂	
21	HHCA	3156	Feather	Feather	87	02/11/11	Homoduplex	♂	
22	HHCB	3157	Feather	Feather	88	02/11/11	Heteroduplex	♀	
23	HHCC	3158	Feather	Feather	71	06/02/10	Homoduplex	♂	
24	HHCF	3160	Feather	Feather	65	06/02/10	Homoduplex	♂	
25	HHCH	3161	Feather	Feather	89	02/11/11	Heteroduplex	♀	
26	HHCJ	3162	Feather	Feather	90	02/11/11	Homoduplex	♂	
27	HHCN	3164	Feather	Feather	91	02/11/11	Heteroduplex	♀	
28	HHFA	n/a	Feather	Feather	92	02/11/11	Heteroduplex	♀	
29	HHFB	3183	Feather	Feather	93	02/11/11	Heteroduplex	♀	
30	HHFC	3188	Feather	Feather	94	02/11/11	Heteroduplex	♀	
31	HHFD	3186	Feather	Feather	95	02/11/11	Homoduplex	♂	
32	HHFF	3190	Feather	Feather	96	02/11/11	Heteroduplex	♀	
33	HHFH	3185	Feather	Feather	25	06/15/10	Heteroduplex	♀	
34	HHFJ	3189	Feather	Feather	21	06/15/10	Heteroduplex	♀	
35	HHFL	3187	Feather	Feather	9	06/03/10	Heteroduplex	♀	
36	HHFN	3198	Feather	Feather	1	02/11/11	Heteroduplex	♀	
37	HHFP	3184	Feather	Feather	2	02/11/11	Homoduplex	♂	
38	HHFS	3229	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
39	HHFT	3197	Feather	Feather	n/a	03/31/11	Homoduplex	♂	

Table A-1 continued

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
40	HHFX	3224	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
41	HHFZ	3222	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
42	HHHA	2926	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	
43	HHHB	2927	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
44	HHHD	2929	Feather	Feather	93	06/03/10	Homoduplex	♂	1.4g
45	HHHF	2930	Feather	Feather	90	06/03/10	Homoduplex	♂	
46	HHHH	2931	Feather	Feather	91	06/03/10	Heteroduplex	♀	
47	HHHJ	2932	Feather	Feather	35	06/15/10	Homoduplex	♂	1.8g
48	HHHL	2933	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
49	HHHN	2934	Feather	Feather	92	06/03/10	Heteroduplex	♀	
50	HHHS	2936	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
51	HHHT	2937	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
52	HHHV	2938	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
53	HHHX	2939	Feather	Feather	10	06/03/10	Heteroduplex	♀	
54	HHHZ	2940	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	
55	HHJA	2941	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
56	HHJB	2942	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
57	HHJC	2943	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	Feather repeat
58	HHJD	2944	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
59	HHJF	2945	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
60	HHJH	2946	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
61	HHJN	2949	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	Blood & Feather repeat
62	HHJP	2950	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
63	HHJS	2951	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
64	HHJT	2952	Feather	Feather	n/a	03/31/11	Homoduplex	♂	Feather repeat
65	HHJV	2953	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
66	HHJX	2954	Feather	Feather	4	06/03/10	Homoduplex	♂	
67	HHJX	2954	Feather	Feather	23	06/15/10	Homoduplex	♂	
68	HHJZ	2955	Feather	Feather	n/a	04/12/11	Homoduplex	♂	
69	HHLA	2956	Feather	Blood	n/a	03/31/11	Homoduplex	♂	
70	HHLB	2957	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
71	HHLC	2958	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
72	HHXX	2959	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
73	HHXZ	2960	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
74	HHZA	2961	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
75	HHZB	2962	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
76	HHZC	2963	Feather	Feather	34	06/15/10	Homoduplex	♂	
77	HHZD	2964	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	
78	HHZF	2965	Feather	Feather	68	06/02/10	Heteroduplex	♀	
79	HHZH	2966	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	

Table A-1 continued

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
80	HHZJ	2967	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
81	HHZL	2968	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
82	HHZN	2969	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
83	HHZP	2970	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
84	HHZS	2971	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
85	HHZT	2972	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
86	HHZV	2973	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
87	HHZX	2974	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
88	HHZZ	2975	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
89	HJAA	2976	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
90	HJAB	2977	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
91	HJAC	2978	Feather	Feather	28	06/15/10	Heteroduplex	♀	
92	HJAD	2979	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
93	HJAF	2980	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
94	HJAH	2981	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
95	HJAJ	2982	Feather	Feather	32	06/15/10	Homoduplex	♂	
96	HJAN	2984	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
97	HJAX	2989	Feather	Feather	76	06/02/10	Homoduplex	♂	
98	HJBB	2992	Feather	Feather	72	06/02/10	Heteroduplex	♀	
99	HJBC	2993	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
100	HJBD	2994	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
101	HJBF	2995	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
102	HJBH	2996	Feather	Feather	73	06/02/10	Heteroduplex	♀	
103	HJBJ	2997	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
104	HJBL	2998	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
105	HJCA	3014	Feather	Feather	n/a	03/31/11	Homoduplex	♂	Feather repeat
106	HJCB	3013	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
107	HJCC	3012	Blood, Feather	Feather	2	06/03/10	Homoduplex	♂	
108	HJCD	3011	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
109	HJCF	3010	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
110	HJCH	3009	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
111	HJCJ	3008	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	
112	HJCL	3007	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	
113	HJCN	3006	Feather	Feather	n/a	03/31/11	Homoduplex	♂	Blood repeat
114	HJCP	3005	Feather	Feather	n/a	03/31/11	Homoduplex	♂	Pen ink, Feather repeat
115	HJCS	3004	Blood, Feather	Blood	n/a	03/31/11	Heteroduplex	♀	
116	HJCT	3003	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
117	HJCV	3002	Feather	Feather	n/a	03/31/11	Homoduplex	♂	1.9g
118	HJCX	3001	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
119	HJCZ	3101	Feather	Feather	n/a	03/31/11	Homoduplex	♂	

Table A-1 continued

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
120	HJDA	3102	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	Repeat
121	HJDB	3103	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
122	HJDC	3104	Blood, Feather	Feather	19	06/15/10	Homoduplex	♂	
123	HJDF	3106	Blood, Feather	Blood	n/a	03/31/11	Heteroduplex	♀	
124	HJDH	3107	Feather	Feather	n/a	04/12/11	Homoduplex	♂	Feather & Blood repeat
125	HJDL	3109	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	Feather repeat, 1.12g
126	HJDN	3110	Feather	Feather	n/a	03/31/11	Homoduplex	♂	Feather repeat
127	HJDP	3111	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
128	HJDS	3112	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
129	HJDT	3113	Feather	Feather	94	06/03/10	Homoduplex	♂	
130	HJDV	3114	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
131	HJDX	3115	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	
132	HJDZ	3116	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
133	HJFA	3117	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	Feather repeat, 1.16g
134	HJFB	3118	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
135	HJFC	3119	Blood, Feather	Feather	79	06/02/10	Homoduplex	♂	
136	HJFD	3120	Feather	Feather	24	06/15/10	Heteroduplex	♀	
137	HJFF	3121	Blood, Feather	Blood	59	06/02/10	Homoduplex	♂	Feather repeat
138	HJFH	3122	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
139	HJFJ	3123	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	Feather repeat
140	HJFN	3125	Blood, Feather	Blood	n/a	03/31/11	Homoduplex	♂	
141	HJFS	3202	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
142	HJFT	3203	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
143	HJFV	3204	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
144	HJFX	3205	Feather	Feather	n/a	03/31/11	Heteroduplex	♀	
145	HJJA	3207	Feather	Feather	n/a	03/31/11	Homoduplex	♂	
146	HJJB	3208	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	Feather repeat
147	HJJC	3209	Blood, Feather	Blood	n/a	04/05/11	Homoduplex	♂	
148	HJJD	3210	Blood, Feather	Feather	7	06/03/10	Homoduplex	♂	
149	HJJF	3211	Feather	Feather	n/a	n/a	Homoduplex	♂	
150	HJJJ	3213	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
151	HJJL	3214	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
152	HJJN	3215	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
153	HJJS	3236	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
154	HJJT	3237	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
155	HJJV	3252	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
156	HJJX	3245	Feather	Feather	30	06/15/10	Heteroduplex	♀	
157	HJJZ	3238	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
158	HJLA	3243	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
159	HJLC	3246	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
160	HJXX	3248	Feather	Feather	96	06/03/10	Homoduplex	♂	

Table A-1 continued

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
161	HJXZ	3249	Feather	Feather	88	06/03/10	Homoduplex	♂	
162	HJZA	3242	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
163	HJZB	n/a	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
164	HJZC	3240	Blood, Feather	Blood	n/a	04/05/11	Heteroduplex	♀	
165	HJZD	3247	Feather	Feather	87	06/03/10	Homoduplex	♂	
166	HJZF	3251	Feather	Feather	1	06/03/10	Heteroduplex	♀	Feather repeat
167	HJZJ	3250	Blood, Feather	Feather	84	06/03/10	Homoduplex	♂	
168	HJZL	3239	Feather	Feather	86	06/03/10	Homoduplex	♂	
169	HJZN	3217	Feather	Feather	n/a	04/05/11	Homoduplex	♂	Feathers & Blood repeat, 1.11g
170	HJZP	3218	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
171	HJZS	3219	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
172	HJZT	3220	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
173	HJZV	3221	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
174	HJZX	3222	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
175	HJZZ	3223	Feather	Feather	67	06/02/10	Homoduplex	♂	
176	HLAA	3257	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
177	HLAB	3244	Blood, Feather	Feather	60	06/02/10	Homoduplex	♂	
178	HLAC	3256	Blood, Feather	Blood	n/a	04/05/11	Heteroduplex	♀	
179	HLAD	3255	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
180	HLAH	3259	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
181	HLAJ	3262	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
182	HLAL	3253	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
183	HLAN	3261	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
184	HLAT	3267	Blood, Feather	Blood	n/a	04/05/11	Homoduplex	♂	
185	HLAV	3266	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
186	HLAX	3265	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
187	HLAZ	3268	Feather	Feather	29	06/15/10	Homoduplex	♂	
188	HLBA	3263	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
189	HLBB	3270	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
190	HLBC	3269	Feather	Feather	20	06/15/10	Homoduplex	♂	
191	HLBD	3277	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
192	HLBF	3275	Blood, Feather	Feather	3	06/03/10	Heteroduplex	♀	
193	HLBL	3272	Feather	Feather	n/a	04/05/11	Homoduplex	♂	Feather repeat
194	HLBN	3276	Feather	Feather	75	06/02/10	Heteroduplex	♀	
195	HLBS	3279	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
196	HLBT	3280	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
197	HLBX	3271	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
198	HLBZ	3287	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
199	HLCA	3285	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
200	HLCB	3286	Feather	Feather	78	06/02/10	Heteroduplex	♀	

Table A-1 continued

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
201	HLCC	3288	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
202	HLCD	3282	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
203	HLCJ	3291	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
204	HLCL	3292	Feather	Feather	26	06/15/10	Heteroduplex	♀	
205	HLCN	3293	Feather	Feather	69	06/02/10	Homoduplex	♂	
206	HLCP	3284	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
207	HLCS	3281	Feather	Feather	27	06/15/10	Homoduplex	♂	
208	HLCT	3306	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
209	HLCV	3307	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
210	HLCZ	3305	Feather	Feather	74	06/02/10	Homoduplex	♂	
211	HLDA	3303	Feather	Feather	70	06/02/10	Homoduplex	♂	
212	HLDB	3301	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
213	HLDC	3296	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
214	HLDD	3302	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
215	HLDF	3298	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
216	HLDH	3299	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
217	HLDJ	3300	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
218	HLDL	3295	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
219	HLDN	3294	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
220	HLDP	3310	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
221	HLDS	3310	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
222	HLDT	3297	Feather	Feather	66	06/02/10	Heteroduplex	♀	
223	HLFA	2853	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
224	HLFB	2900	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
225	HLFC	2901	Feather	Feather	n/a	04/05/11	Homoduplex	♂	
226	HLFD	2898	Feather	Feather	n/a	04/05/11	Heteroduplex	♀	
227	HLFH	2897	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
228	HLFJ	2894	Blood, Feather	Feather	61	06/02/10	Heteroduplex	♀	
229	HLFL	2904	Feather	Feather	n/a	n/a	Heteroduplex	♀	
230	HLFN	2893	Feather	Feather	n/a	n/a	Homoduplex	♂	Feather repeat
231	HLFP	2892	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
232	HLFS	2910	Feather	Feather	n/a	n/a	Heteroduplex	♀	
233	HLFV	2899	Feather	Feather	n/a	n/a	Homoduplex	♂	
234	HLFX	2905	Feather	Feather	n/a	n/a	Heteroduplex	♀	
235	HLFZ	2989	Feather	Feather	n/a	n/a	Homoduplex	♂	
236	HLHA	2868	Feather	Feather	n/a	n/a	Heteroduplex	♀	
237	HLHD	2885	Feather	Feather	n/a	n/a	Homoduplex	♂	
238	HLHF	2877	Feather	Feather	n/a	n/a	Homoduplex	♂	
239	HLHH	2869	Feather	Feather	n/a	n/a	Heteroduplex	♀	
240	HLHJ	2870	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
241	HLHL	2884	Feather	Feather	n/a	n/a	Homoduplex	♂	
242	HLHN	2877	Feather	Feather	n/a	n/a	Heteroduplex	♀	
243	HLHP	2875	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	

Table A-1 continued

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
244	HLHS	2886	Feather	Feather	n/a	n/a	Heteroduplex	♀	
245	HLHX	2871	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
246	HLJA	2851	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
247	HLJB	2858	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
248	HLJC	2857	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
249	HLJD	2859	Blood, Feather	Blood	57	06/02/10	Heteroduplex	♀	
250	HLJJ	2856	Blood, Feather	Blood	n/a	04/12/11	Heteroduplex	♀	
251	HLJL	2862	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
252	HLJN	2854	Blood, Feather	Feather	64	06/02/10	Heteroduplex	♀	Blood repeat
253	HLJZ	2852	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
254	HLLA	2873	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
255	HLLB	2888	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
256	HLXZ	2759	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	Mislabeled (Ninos y crias code list)? HLJP?
257	HLZA	2882	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
258	HLZC	2923	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
259	HLZD	2907	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
260	HLZF	2887	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
261	HLZH	2908	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
262	HLZJ	3092	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
263	HLZL	2909	Blood, Feather	Blood	n/a	n/a	n/a	n/a	No amp.
264	HLZN	2880	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
265	HLZP	2878	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
266	HLZT	2916	Feather	Feather	n/a	04/12/11	Heteroduplex	n/a	
267	HLZV	2906	Feather	Feather	n/a	04/12/11	Heteroduplex	n/a	
268	HNAL	2787	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
269	HNAP	2726	Feather	Feather	58	05/26/11	Homoduplex	♂	
270	HNAT	2723	Blood, Feather	Blood	n/a	n/a	n/a	n/a	No amp.
271	HNAV	2729	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
272	HNAX	2727	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
273	HNAZ	2911	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
274	HNBA	2730	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
275	HNBB	2789	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
276	HNBC	n/a	Feather	Feather	n/a	04/12/11	Heteroduplex	♀	
277	HNBH	2834	Feather	Feather	95	06/03/10	Heteroduplex	♀	
278	HNBL	n/a	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
279	HNBP	2818	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
280	HNBS	2722	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
281	HNBV	n/a	Blood, Feather	Feather	n/a	n/a	n/a	n/a	No amp.
282	HNBX	3093	Blood, Feather	Blood	n/a	n/a	n/a	n/a	No amp.
283	HNCA	2841	Blood, Feather	Blood	n/a	n/a	n/a	n/a	No amp.
284	HNCB	2728	Feather	Feather	n/a	n/a	n/a	n/a	No amp.

Table A-1 continued

	Plastic band	Metal ring	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
285	HNCH	n/a	Feather	Feather	n/a	n/a	n/a	n/a	Feather repeat, 1.15g, No amp.
286	HNCJ	2827	Blood, Feather	Blood	n/a	n/a	n/a	n/a	No amp.
287	HNCL	2790	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
288	HNCP	2820	Feather	Feather	n/a	n/a	n/a	n/a	No amp.
289	HNCT	2822	Feather	Feather	67	06/02/11	Homoduplex	♂	
290	HNDA	2819	Feather	Feather	68	06/02/11	Heteroduplex	♀	
291	HNDD	2785	Feather	Feather	69	06/02/11	Heteroduplex	♀	Rescored
292	HNDT	3100	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	Rescored
293	HNFF	n/a	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
294	HNFL	3098	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
295	HNFN	2915	Feather	Feather	70	06/02/11	Homoduplex	♂	
296	HNFP	3090	Feather	Feather	59	05/26/11	Heteroduplex	♀	
297	HNFS	2924	Feather	Feather	60	05/26/11	Heteroduplex	♀	
298	HNFT	2913	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
299	HNFV	2919	Feather	Feather	72	06/02/11	Homoduplex	♂	
300	HNFZ	3091	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
301	HNHA	2925	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
302	HNHD	2912	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
303	HNHF	2920	Feather	Feather	n/a	n/a	n/a	n/a	Pen ink, Blood & Feather repeat, No amp.
304	HNHL	3015	Feather	Feather	n/a	n/a	n/a	n/a	Pen ink, Feather repeat, No amp.
305	HNHP	3017	Blood, Feather	Blood	n/a	n/a	n/a	n/a	Pen ink, Feather & Blood repeat, No amp.
306	HNHT	3019	Blood, Feather	Blood	73	06/02/11	Homoduplex	♂	
307	HNHV	3020	Blood, Feather	Blood	74	06/02/11	Heteroduplex	♀	Feather repeat
308	HNHX	3031	Feather	Feather	n/a	n/a	n/a	n/a	1.7g
309	HNJD	3026	Blood, Feather	Blood	n/a	n/a	n/a	n/a	No amp.
310	HNJP	3032	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
311	HNJS	3033	Feather	Feather	n/a	n/a	n/a	n/a	No amp., 1.1g
312	HNJT	3034	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
313	HNJV	3035	Blood, Feather	Blood	n/a	05/26/11	Homoduplex	♂	
314	HNJX	3036	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	

Table A-1 continued

	Plastic band	Metal band	Tissue Type	Tissue Isolated	Capillary # (HRM)	Date (MM/DD/YY)	Gentoype (HRM)	Gender (HRM)	Remarks/Other info. From Labeled Bags
315	HNJY	n/a	Feather	n/a	n/a	n/a	n/a	n/a	
316	HNJZ	3037	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
317	HNLA	3038	Blood, Feather	Blood	n/a	05/26/11	Heteroduplex	♀	Mislabeled (Ninos y crias code list)
318	HNLB	3039	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
319	HNLC	3040	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
320	HNXX	3041	Feather	Feather	12	06/15/10	Homoduplex	♂	
321	HNXZ	3042	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
322	HNZA	3043	Feather	Feather	18	06/15/10	Homoduplex	♂	
323	HNZB	3044	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
324	HNZC	3045	Blood, Feather	Blood	n/a	05/26/11	Heteroduplex	♀	
325	HNZD	3046	Feather	Feather	33	06/15/10	Heteroduplex	♀	
326	HNZN	3050	Blood, Feather	Blood	n/a	05/26/11	Homoduplex	♂	
327	HPAJ	3063	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
328	HPAP	3066	Feather	Feather	14	06/15/10	Homoduplex	♂	
329	HPAS	3067	Feather	Feather	16	06/15/10	Heteroduplex	♀	
330	HPAT	3068	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
331	HPAX	3070	Feather	Feather	77	06/02/10	Homoduplex	♂	
332	HPAZ	3071	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
333	HPBA	3072	Feather	Feather	n/a	n/a	n/a	n/a	Mislabeled (Ninos y crias code list)
334	HPBB	3073	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
335	HPBC	3074	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
336	HPBD	3075	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
337	HPBF	3076	Feather	Feather	15	06/15/10	Homoduplex	♂	
338	HPBH	3077	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
339	HPBJ	3078	Feather	Feather	13	06/15/10	Heteroduplex	♀	
340	HPBL	3079	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
341	HPBN	3080	Feather	Feather	5	06/03/10	Heteroduplex	♀	
342	HPBP	3081	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
343	HPBS	3082	Feather	Feather	17	06/15/10	Heteroduplex	♀	
344	HPBT	3083	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
345	HPBV	3084	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
346	HPBX	3085	Feather	Feather	8	06/03/10	Heteroduplex	♀	
347	HPHD	2750	Feather	Feather	61	05/26/11	Heteroduplex	♀	
348	HPHF	n/a	Feather	Feather	62	05/26/11	Homoduplex	♂	Mislabeled (Ninos y crias code list)
349	HPHH	2752	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
350	HPHJ	2890	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
351	HPHL	2891	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
352	HPHN	2756	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
353	HPHP	2551	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
354	HPHS	2755	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
355	HPHT	2754	Feather	Feather	n/a	05/26/11	Homoduplex	♂	
356	HPHV	2753	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
357	HPJA	n/a	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	Rescored
358	HPJB	n/a	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	
359	HPJC	n/a	Feather	Feather	n/a	05/26/11	Heteroduplex	♀	

APPENDIX B

Table B-1 GenBank Accession numbers. Sequences were generated from amplified products targeting the Z chromosome of males using primers P2 R and P14 F and W chromosome using primers P2 R and P0 F.

Species	Common name	CHD allele	Genbank Accession #
Ephippiorhynchus senegalensis	Saddle-billed Stork	W	submitted
Ephippiorhynchus senegalensis	Saddle-billed Stork	Z	submitted
Eudocimus ruber	Scarlet Ibis	W	submitted
Eudocimus ruber	Scarlet Ibis	Z	submitted
Leptoptilos crumenifer	Marabou Stork	Z	submitted
Ciconia abdimii	White-bellied Stork	W	submitted
Ciconia abdimii	White-bellied Stork	Z	submitted
Phoenicopterus chilensis	Chilean Flamingo	W	submitted
Phoenicopterus chilensis	Chilean Flamingo	Z	submitted
Platalea ajaja	Roseate Spoonbill	Z	submitted
Phoenicopterus minor	Lesser Flamingo	W	submitted
Phoenicopterus minor	Lesser Flamingo	Z	submitted
Tympanuchus cupido attwateri	Attwater's Prairie Chicken	W	submitted
Tympanuchus cupido attwateri	Attwater's Prairie Chicken	Z	submitted

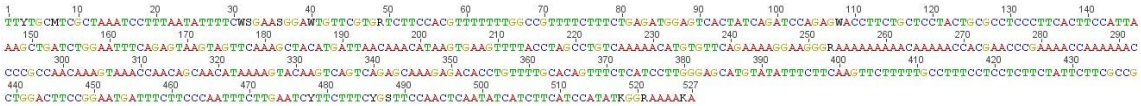

Figure B-1 Sequence of the CHD-Z amplified segment of Saddle-billed Stork (*Ephippiorhynchus senegalensis*). Sequence was generated using primers P2 and P14 (Griffiths et al. 1998).

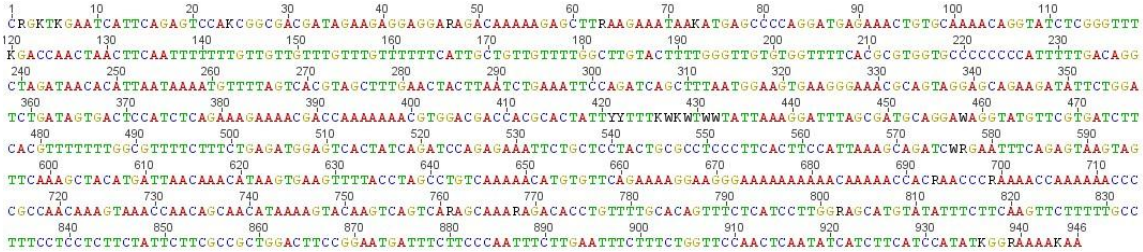

Figure B-2 Sequence of the CHD-W amplified segment of Saddle-billed Stork (*Ephippiorhynchus senegalensis*). Sequence was generated using primers P2 (Griffiths et al. 1998) and P0 (Han et al. 2009).

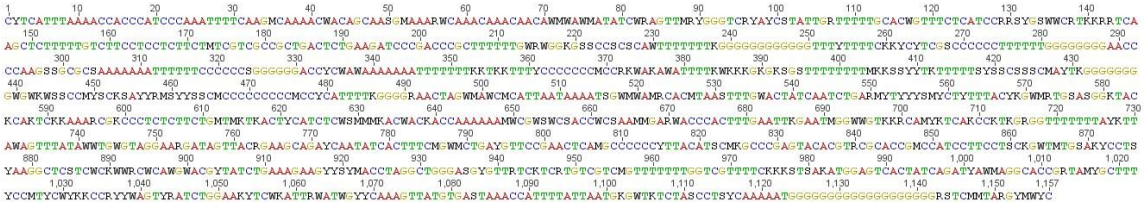

Figure B-3 Sequence of the CHD-W amplified segment of Scarlet Ibis (*Eudocimus ruber*). Sequence was generated using primers P2 (Griffiths et al. 1998) and P0 (Han et al. 2009).

Figure B-4 Sequence of the CHD-Z amplified segment of Scarlet Ibis (*Eudocimus ruber*). Sequence was generated using primers P2 and P14 (Griffiths et al. 1998).

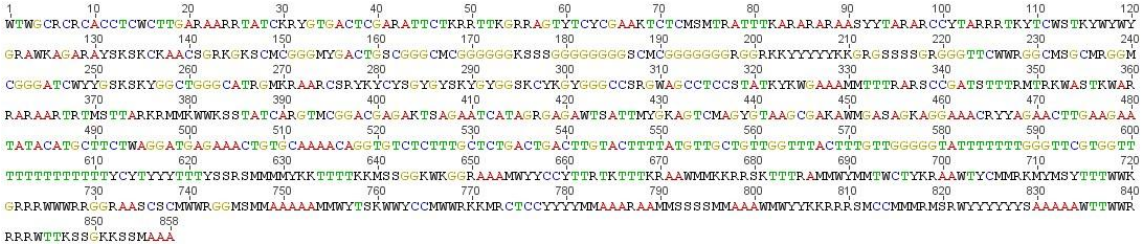

Figure B-5 Sequence of the CHD-Z amplified segment of Marabou Stork (*Leptoptilos crumeniferus*). Sequence was generated using primers P2 and P14 (Griffiths et al. 1998).

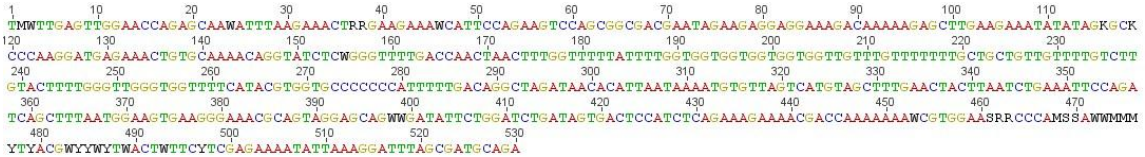

Figure B-6 Sequence of the CHD-W amplified segment of Chilean Flamingo (*Phoenicopterus chilensis*). Sequence was generated using primers P2 (Griffiths et al. 1998) and P0 (Han et al. 2009).

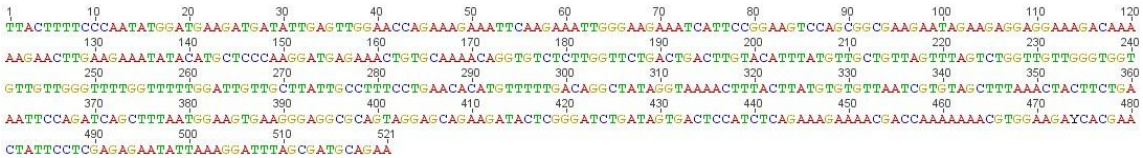

Figure B-7 Sequence of the CHD-Z amplified segment of Chilean Flamingo (*Phoenicopterus chilensis*). Sequence was generated using primers P2 and P14 (Griffiths et al. 1998).

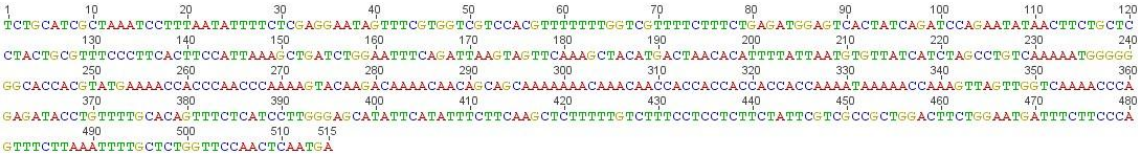

Figure B-8 Sequence of the CHD-W amplified segment of Lesser Flamingo (*Phoenicopterus minor*). Sequence was generated using primers P2 (Griffiths et al. 1998) and P0 (Han et al. 2009).

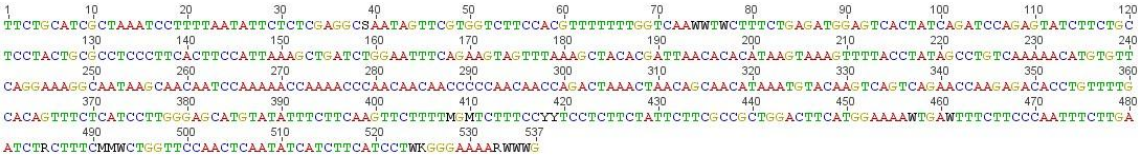

Figure B-9 Sequence of the CHD-Z amplified segment of Lesser Flamingo (*Phoenicopterus minor*). Sequence was generated using primers P2 and P14 (Griffiths et al. 1998).

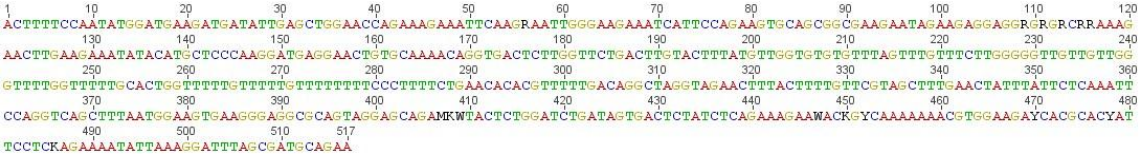

Figure B-10 Sequence of the CHD-Z amplified segment of Roseate Spoonbill (*Platalea ajaja*). Sequence was generated using primers P2 and P14 (Griffiths et al. 1998).

Figure B-11 Sequence of the CHD-W amplified segment of Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*). Sequence was generated using primers P2 (Griffiths et al. 1998) and P0 (Han et al. 2009).

Figure B-12 Sequence of the CHD-Z amplified segment of Attwater's Prairie Chicken (*Tympanuchus cupido attwateri*). Sequence was generated using primers P2 and P14 (Griffiths et al. 1998).